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NAME OF AUTHOR/*NOM DE L'AUTEUR* MARY T FAIRHURST

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NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* D. L. Rabenstein.

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DATED/*DATÉ* Feb. 24, 1975. SIGNED/*SIGNÉ* Mary Fairhurst

PERMANENT ADDRESS/*RÉSIDENCE FIXE* 1150 Crevier St.
Montreal, Quebec.
H4L 2W7

NL-91 (3-74)

THE UNIVERSITY OF ALBERTA

NUCLEAR MAGNETIC RESONANCE STUDIES OF THE SOLUTION CHEMISTRY OF METHYLMERCURY AND THE ACID-BASE CHEMISTRY OF AMINO ACIDS

by

© MARY TERESA FAIRHURST

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled, NUCLEAR MAGNETIC RESONANCE STUDIES OF THE SOLUTION CHEMISTRY OF METHYL-MERCURY AND THE ACID-BASE CHEMISTRY OF AMINO ACIDS, submitted by MARY TERESA FAIRHURST in partial fulfilment of the requirements for the degree of Doctor of PHILOSOPHY.

D.L. Rabenstein,
(Supervisor).

B. Kratochvil.

R.E.D. McClung.

G. Horlick.

L.G. Chatten.

A. Corsini,
(External Examiner).

Date: February 11, 1975.

To My Parents

ABSTRACT

Part 1

The aqueous solution chemistry of the methylmercury complexes of several amino acids and the peptide glutathione has been investigated by proton and carbon-13 magnetic resonance spectroscopy. In an amino acid such as valine the nature of the complex formed is pH dependent. At low pH protons compete with methylmercury for the ligand while at high pH hydroxide ions compete with the ligand for methylmercury. At low pH the carboxylic acid group is the binding site in valine while at higher pH the methylmercury is bound by the amino group. Formation constants are determined for both these complexes. Results obtained for methionine indicate for the first time significant binding of methylmercury by the thioether group. The methylmercury leaves the thioether group and migrates to the amino group at high pH. The formation constants for the different complexes are determined. Binding of methylmercury by cysteine, penicillamine and glutathione was investigated over a variety of solution conditions. In solutions containing equimolar amounts of methylmercury and ligand, methylmercury binds most strongly to the ionized sulfhydryl group, with no detectable dissociation of the complex over the pH range 0-14. Evidence is presented for some protonation of the methylmercury-complexed sulfhydryl group of glutathione at pH < 2. In solutions con-

taining two moles of methylmercury for each mole of glutathione, both methylmercury cations are bound to the sulfhydryl group up to pH 4, from pH 4-8, one methylmercury shifts from the sulfhydryl group to the amino group, while above pH 10 it dissociates to form CH_3HgOH.

The kinetics of the ligand exchange reactions of the methylmercury-methylamine and methylmercury-glutathione complexes were studied by NMR line-broadening techniques. Of the possible pathways by which methylmercury can exchange between the free and complexed forms in the methylmercury-methylamine system, first order dissociation is the only pathway contributing significantly to the methylmercury exchange over the pH range 2 to 5. The rate constant for this reaction was determined to be 70 ± 18 sec^{-1}. For the methylmercury-glutathione system the major pathway for ligand exchange depends on the solution pH: below pH 2 ligand exchange occurs mainly by means of a proton-assisted dissociation of the complex while above pH 2 the exchange takes place by displacement of complexed glutathione by sulfhydryl-deprotonated glutathione. The rate constants for these two reactions were determined to be 600 ± 200 m^{-1} sec^{-1} and $5.8 \pm 1.9 \times 10^8$ m^{-1} sec^{-1}. The mechanisms of the ligand exchange reactions are discussed.

Part 2

The application of carbon-13 magnetic resonance spectroscopy to the characterization of the acid-base chemistry of amino acids at the molecular level was studied using the amino acids cysteine and penicillamine and the related compound 2-aminoethanethiol. Chemical shift titration curves for each of the carbon atoms of these three compounds were obtained. From each of these curves macroscopic acid dissociation constants for the overlapping amino acid and sulfhydryl deprotonations were evaluated.

Equations are presented which relate the observed chemical shifts to the microscopic dissociation constants for such compounds. It is shown that, to evaluate the microscopic dissociation constants, one needs to know the chemical shift of each carbon atom in each of the forms present. Attempts to obtain these shifts using chemical shift data from model compounds were unsuccessful presumably due to the dependence of the carbon-13 chemical shift on the conformation of the compound. The chemical shift versus pH curves of penicillamine are discussed in terms of the likely conformations of penicillamine.

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PART 1

NUCLEAR MAGNETIC RESONANCE STUDIES OF THE SOLUTION CHEMISTRY OF METHYLMERCURY

INTRODUCTION

The chemistry of the methylmercury cation, $CH_3Hg(II)$, has been the subject of considerable research, partly due to its unique properties (1). It is a metallic species with a coordination number of one (2), it is large, highly polarizable and the simplest soft acid (2,3), and its complexation reactions may proceed via an associative mechanism (4) in contrast to the dissociative mechanism characteristic of most other metal complexation reactions (5). Because of its affinity for sulfur, $CH_3Hg(II)$ has been used as a highly selective reagent for protein sulfhydryl groups for some time (6). More recently, it has been suggested as a chemical probe for unpaired bases in superhelical DNA (7).

Another dimension of interest in the chemistry of methylmercury has been added by the recent discovery that methylmercury can be formed from inorganic mercury by microbiological processes (8,9,10). Kurland, et. al. (8) suggested that mercury could be alklyated by plankton and other marine life. In 1968 Wood and coworkers (9) demonstrated that both methylmercury and dimethylmercury are formed from Hg(II) in methanogenic bacteria. In 1971, these same workers elucidated reaction pathways for both enzymatic and non-enzymatic methylation of mercury, the non-enzymatic methylation proceeding via an electrophilic

attack by Hg(II) on methylcobalamin and suggested that any microorganism capable of synthesizing methylcobalamin can synthesize methylmercury (10). These studies explain the observation that the mercury in victims of several mercury poisoning epidemics was in the form of methylmercury, even though the industrial wastes thought to be responsible for the mercury pollution contained inorganic mercury (11,12). Methylmercury has been shown to be the most toxic form of mercury, forming water soluble compounds which attack the central nervous system, essentially irreversibly.

Since methylmercury does not exist as CH_3Hg^+ but rather within a coordination sphere of water, as $CH_3HgOH_2^+$, a knowledge of its coordination chemistry is likely to be useful in understanding the behaviour of methylmercury in the environment and in biological systems.

In part I of this thesis, the results of nuclear magnetic resonance (NMR) studies of the binding of methylmercury by the nitrogen, oxygen and sulfur groups of selected amino acids and peptides are described.

A. The Aqueous Solution Chemistry of Methylmercury

The first work on the aqueous solution chemistry of methylmercury was reported in 1953 by Waugh, Walton and Laswick (14) who determined an equilibrium constant for the formation of methylmercuric hydroxide as described

by Equations (1) and (2).

$$CH_3HgOH_2^+ + OH^- \rightleftarrows CH_3HgOH + H_2O \qquad (1)$$

$$K_1 = \frac{[CH_3HgOH]}{[CH_3HgOH_2^+][OH^-]} \qquad (2)$$

From the pH at half neutralization in the titration of methylmercuric hydroxide with nitric acid and perchloric acid, they obtained a value of 3.1×10^9 for K_1. The assumption inherent in this procedure is that the only methylmercury species present during the titration are $CH_3HgOH_2^+$ and CH_3HgOH.

Twelve years later, Schwarzenbach and Schellenberg (2) showed by pH titration that, in addition to the aquated species and methylmercuric hydroxide, a dinuclear complex in which two methylmercury cations are attached to the same hydroxide ligand is also formed. The model developed by Schwarzenbach and Schellenberg for the aqueous solution chemistry of methylmercury is described by Equations (1) - (4).

$$CH_3HgOH + CH_3HgOH_2^+ \rightleftarrows (CH_3Hg)_2OH^+ + H_2O \qquad (3)$$

$$K_2 = \frac{[(CH_3Hg)_2OH^+]}{[CH_3HgOH][CH_3HgOH_2^+]} \qquad (4)$$

The values they obtained for K_1 and K_2 are 2.34×10^9 and

2.34×10^2.

The model developed by Schwarzenbach and Schellenberg has recently been questioned (15,16) on the basis of a report by Grdenic and Zado (17) that trimethylmercurioxonium perchlorate, $[(CH_3Hg)_3O]ClO_4$, precipitates from concentrated neutral solutions prepared by titration of methylmercuric hydroxide with perchloric acid, and their conclusion from synthetic studies that methylmercuric hydroxide does not exist as a chemical compound except possibly in dilute aqueous solution as the dissociation product of trismethylmercurioxonium hydroxide, $[(CH_3Hg)_3O]OH$. These workers claimed to have prepared $[(CH_3Hg)_3O]OH$ by a metathetical reaction between $[(CH_3Hg)_3O]ClO_4$ and KOH in methanol and concluded from conductance data that $(CH_3Hg)_3O^+$ is stable in alkaline (methanol) solution and that the compound previously thought to be CH_3HgOH is actually $[(CH_3Hg)_3O]OH$ or its dehydration product. These results suggest that trismethylmercurioxonium ion, $(CH_3Hg)_3O^+$, might also be present in aqueous solution, in which case the model of Schwarzenbach and Schellenberg is incomplete. In Chapter III, the existence of the trismethylmercurioxonium ion in aqueous solution is considered further and the model of Schwarzenbach and Schellenberg is modified to include this ion also.

B. The Coordination Chemistry of Methylmercury

Although the parent Hg(II) ion forms both two-coordinate linear and four-coordinate tetrahedral complexes (18), the predominant coordination number of mercury in $CH_3Hg(II)$ is two (2). It has been suggested that the coordination number of two is favoured for mercury in $CH_3Hg(II)$ due to a smaller positive charge on the mercury atom in complexes such as CH_3HgCN as compared to $Hg(CN)_2$ (19).

To determine the tendency for higher complex formation, Schwarzenbach and Schellenberg (2) measured the solubility of CH_3HgI in 0.1 M KNO_3 and in 0.1 M KI. They observed a solubility of 1.67×10^{-3} moles/l. in 0.1 M KI compared to 1.41×10^{-3} moles/l in 0.1 M KNO_3 and attributed the increased solubility to formation of $CH_3HgI_2^-$. From these solubility data, they calculated the formation constant for the $CH_3HgI_2^-$ complex to be approximately 2, as compared to 4×10^8 for the formation constant of CH_3HgI. They also reported maximum values of 3 for the formation constants of $CH_3Hg(CN)_2^-$ and $CH_3Hg(SR)_2^-$ as compared to values of 1.3×10^{14} for CH_3HgCN and 1.3×10^{16} for $CH_3HgSCH_2CH_2OH$. These results are consistent with a predominant coordination number of one for $CH_3Hg(II)$.

Simpson (20) observed that the formation constant for CH_3HgCN did not show any systematic change when

the concentration of cyanide was varied over an order of magnitude and also concluded higher complexes are unimportant.

Goggin and Woodward (19) were unable to detect complexes of the type $[CH_3Hg(CN)_n]^{(n-1)-}$, where n is greater than one, by Raman spectroscopy. The Raman spectrum of an aqueous solution of CH_3HgCN containing a three-fold excess of potassium cyanide was identical with that for CH_3HgCN except for an additional intense, strongly polarized line at 2081 cm^{-1} which is due to the free cyanide ion.

Sytsma and Kline (21) considered the question of chelation to methylmercury. The methylmercury complexes of some potential chelating thiophenols were examined by proton magnetic resonance spectroscopy. On the basis of the spin-spin coupling constants for coupling between the methyl protons and the mercury-199 nucleus, they concluded that methylmercury is bonded only to the sulfur atoms of the thiophenol.

The mercury ion found in methylmercury complexes is linearly coordinated by the methyl group and another ligand (19,22). The halide and cyanide complexes of methylmercury were found to possess C_{3v} symmetry (19), while a mean C-Hg-S angle of 178(2)° in the D,L-penicillaminato complex of methylmercury was found by X-ray structure determination (22).

The first quantitative work on the formation of methylmercury complexes by nitrogen-, oxygen-, and sulfur-containing ligands was reported by Simpson in 1961 (20). He determined the formation constants by pH methods involving competition between OH^- and X^- for $CH_3Hg(II)$, where X^- is the conjugate base of a strong acid, or competition between H^+ and $CH_3Hg(II)$ for B^-, where B^- is the conjugate base of a weak acid, and by polarographic methods. The aqueous solution model he used was based on the model of Waugh, Walton and Laswick which did not include the dimeric species, $(CH_3Hg)_2OH^+$. In some cases, the value obtained for the formation constant depended upon the method used. For example, the logarithm of the formation constant for complexation by the sulfhydryl group of cysteine was found to be 15.7 by the pH method and 16.6 by the polarographic method. The reason for the differences was not given. The other ligands studied by Simpson which are of interest in this thesis were acetic acid, ammonia and glutathione. He reported values of 3.6, 8.4, and 16.5 respectively for the logarithm of the methylmercury complex formation constants of these ligands. In his summary, Simpson states that, of the functional groups present in proteins, methylmercury would bind first to the sulfhydryl group. Of the other potential coordination sites, the imidazole and amine groups are most important; at low pH methylmercury

binds equally to imidazole and amine groups while at pH greater than 6, amine binding becomes more important than imidazole binding.

In 1965, Schwarzenbach and Schellenberg reported the formation constants for a variety of methylmercury complexes (2). Some of their results are given in Table I. They pointed out some similarities between the methylmercury cation and the hydrogen ion. Both have a preferred coordination number of one and form complexes having ligand to cation ratios greater than one only at very high ligand concentrations. Both form polynuclear complexes in which more than one cation is attached to the same ligand:

$$L^{\lambda-} \rightarrow CH_3HgL^{1-\lambda} \rightarrow (CH_3Hg)_2L^{2-\lambda} \rightarrow (CH_3Hg)_3L^{3-\lambda}$$

For example, when L is OH^-, the complexes CH_3HgOH and $(CH_3Hg)_2OH^+$ are formed and when L is $S^=$ the complexes CH_3HgS^-, $(CH_3Hg)_2S$, and $(CH_3Hg)_3S^+$ are formed. Schwarzenbach and Schellenberg point out that although the methylmercury cation resembles the proton in the simple stoichiometry of its reactions, its tendency for reaction with various ligands is different. Whereas the stability of the hydrogen halides is HF >> HCl > HBr > HI, the stability of the methylmercury halides is in the opposite order. Also the proton in general has a higher affinity for the oxygen atoms rather than the sulfur

Table I. Formation Constants for Methylmercury Complexes of Various Ligands

Ligand	log K_{F-}
F^-	1.50
Cl^-	5.25
Br^-	6.62
I^-	8.60
S^{2-}	21.2
CH_3HgS	16.3
$(CH_3Hg)_2S$	7
$HOCH_2CH_2\text{-}S^-$ [a]	16.12
$HO\text{-}CH_2\text{-}CH_2\text{-}S\text{-}HgCH_3$ [a]	6.27
NH_3	7.60
$NH_2CH_2CH_2NH_2$	8.25
CN [b]	[illegible].1

[a] coordinated through sulfur

[b] coordinated through carbon

atoms of bases, whereas methylmercury prefers coordination to sulfur atoms. On the basis of these relative affinities, the proton is considered a hard acid, while the large easily polarizable methylmercury cation is stoichiometrically the simplest soft acid (3).

The binding of methylmercury by nucleosides was studied by Simpson (23). Using absorption spectroscopy he determined the nitrogen sites to which methylmercury binds over the pH range 0 - 14 for the 5 naturally-occurring nucleosides. More recently, Mansy, Wood, Sprowles, and Tobias (15) published a more complete study of methylmercury binding to pyrimidine nucleosides and nucleotides using Raman Difference Spectroscopy. They concluded that methylmercury binds preferentially to the most basic site, in this case to one of the deprotonated ring nitrogens in preference to an oxygen site. Gruenwedel and Davidson (24) assumed methylmercury binds to the deprotonated nitrogen sites on the nucleotides in a DNA structure. Since the protons displaced by the $CH_3Hg(II)$ in this way are involved in Watson-Crick hydrogen bonding, the methylmercury causes denaturation of the native DNA. With the exception of Mansy, et. al. (15), the formation of polynuclear hydroxy complexes of methylmercury has not been taken into account in the study of the binding of methylmercury by nucleosides and nucleotides. Gruenwedel and Davidson assumed that at pH 5, all

methylmercury not complexed by nucleoside or nucleotide is present as methylmercuric hydroxide. The results of Schwarzenbach and Schellenberg indicate that this is not the case even at relatively low total concentrations of methylmercury.

The complexation of methylmercury by several sulfur-containing ligands has been reported (25,22,26,28, 29). Goggin and Woodward (25) showed that in 1.3 M aqueous solution prepared from methylmercuric nitrate, dimethylsulphide successfully competes with nitrate ions and water molecules for $CH_3Hg(II)^+$ ions to form $CH_3Hg\text{-}S(CH_3)_2^+$. The X-ray structure of solid D,L-penicillaminatomethylmercury shows clearly that the penicillamine is in a zwitterion form and is bonded to the methylmercury via a deprotonated sulfhydryl group (22). There was no evidence for any intra- or intermolecular coordination of N or O atoms to the mercury atom. The interaction of methylmercury with metallothionein, a sulfhydryl-rich protein, has been investigated (26). Somewhat surprisingly, thionein has a lower affinity for methylmercury than cadmium, whereas the affinity of glutathione, which contains a single sulfhydryl group, is greater for methylmercury than for cadmium (27).

The binding of methylmercury by the sulfhydryl group of N-acetylcysteine has been studied by Simpson, Hopkins and Hague (28) who concluded that two or more

methylmercury cations can bind to the sulfhydryl group although they did not define the solution conditions under which this would occur. However, Wong, et. al. (29) isolated a 2:1 methylmercury-penicillamine complex from alkaline solution and showed by X-ray crystallography that one methylmercury cation is bound by the sulfhydryl group and the other by the amino group.

Eigen, Geier and Kruse (4) have investigated the dynamics of methylmercury complexation reactions by the temperature jump technique. They determined the forward and reverse rate constants for ligand displacement reactions of the type shown in Equation (5)

$$CH_3HgX + OH^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} CH_3HgOH + X^- \qquad (5)$$

where X^- is Cl^-, Br^-, I^-, CN^-, SCN^- or SO_3^{2-}. They concluded that the methylmercury displacement reactions which they studied proceed via an associative mechanism, that is the reacting ligand displaces the complexed ligand. This is different from the kinetics of most metal ion complexation reactions which are thought to follow a dissociative mechanism, that is, the rate-determining step is the leaving of a water molecule from the inner coordination sphere of the metal ion (5).

It has been demonstrated by proton magnetic resonance (PMR) spectroscopy that the methyl group in methylmercury is nonlabile. The presence of satellite

resonances which arise from spin-spin coupling of the protons of methylmercury to the 16.9% of mercury nuclei which has a spin of 1/2 (Hg-199) in the NMR spectrum proves that there is no dissociation of the mercury-carbon bond. Initially some confusion existed because these satellites are broadened in the iodo- and bromo-methylmercury complexes (30). In early NMR studies this broadening was interpreted in terms of exchange of the methyl groups; however, later work showed that the observed broadening results from quadrupole interactions between the iodine and bromine and the protons and not from methyl group exchange (31). In contrast to methylmercury, the satellites of dimethylmercury collapsed in the presence of aluminum trichloride due to methyl group exchange (32).

In 1967, Simpson (33) measured the displacement of hydroxide ion from the methylmercury hydroxide complex by cyanide using NMR line broadening techniques. His results supported the conclusions concerning the associative reaction mechanism. He also demonstrated that polynuclear species such as $(CH_3Hg)_2OH^+$ are probably not of kinetic importance and predicted that the rate of exchange of methylmercury between unhindered sulfhydryl groups would be too fast to measure by NMR line broadening techniques. Recently Simpson, Hopkins and Hague (28) reported kinetic results obtained by proton magnetic

resonance spectroscopy for the binding of methylmercury chloride by N-acetyl-L-cysteine. They concluded that exchange of the peptide between the free and complexed forms is dominated by a pathway involving first order dissociation of the complex. Due to the spin-spin splitting of the broadened resonances, they were only able to estimate the rate constant for this first order dissociation. Their estimated value is suspect, however, since the rate constant predicted for the reaction of methylmercury and N-acetyl-L-cysteine from their values and the formation constant of the methylmercury-cysteine complex (20) is six to seven orders of magnitude larger than the rate constants for a diffusion-controlled bimolecular reaction.

C. Overview of Part I

In view of the importance of methylmercury and the sparsity of information about its coordination chemistry with amino acid and peptide ligands, it was decided to attempt to characterize more completely the binding of methylmercury by amino acids and peptides. On the basis of results reported for NMR studies of methylmercury compounds, nuclear magnetic resonance spectroscopy was chosen as the principal technique for this study.

Both the chemical shift of the methyl protons and the mercury-proton spin-spin coupling constant in

CH_3HgX compounds depend on the nature of X as illustrated by the results of Hatton, et. al. (30) given in Table II. These results indicate that the absolute value of the spin-spin coupling constant decreases as the substituent is made more electronegative. In the case of the nitrate and perchlorate complexes they recognized that the species being observed may be $CH_3HgOH_2^+$. The dependence of the spin-spin coupling constant on the nature of X is further illustrated by the linear correlation observed between spin-spin coupling constant and the aqueous solution pK_A of the carboxylic acid in a series of methylmercury carboxylates in chloroform solution (34). In a study of the binding of methylmercury by selected carboxylic acids in aqueous solution it was found that the absolute magnitude of the spin-spin coupling constant decreases approximately linearly as both the pK_A of the ligand and the log K_P of the complex increase (35). Sytsma and Kline (21) also observed a linear relationship between the spin-spin coupling constant and pK_A for the ligand which they attributed to the fact that both the spin-spin coupling constant and pK_A are related to the polarizability of the basic site to which the proton and methylmercury are bonded.

Variation in the spin-spin coupling constant has also been attributed to changes in the s-character of the mercury-carbon bond. Federov, Kalinin, Gasanov, and Zakharkin (36) observed that solvation caused an increase

Table II. Chemical Shift of the Methyl Protons and the Mercury-Proton Coupling Constant of Methyl-Mercury in Methylmercury Complexes.

Complex	δ_{CH_3} [a]	J_{CH_3Hg} [b]
CH_3HgCN	0.650	178.0
CH_3HgOAc	0.525	220.8
CH_3HgOH	0.442	214.2
CH_3HgCl	0.425	215.2
CH_3HgNO_3	0.362	227.0
CH_3HgClO_4	0.170	233.2

[a] ppm vs. cyclohexane

[b] Hz

in the spin-spin coupling constant. Goggin, Goodfellow, Haddock and Eary (37) also found that the spin-spin coupling constant depends on the ligand and increases in the following order: CH_3^- < Ph^- << PMe_3 < CN^- ≈ $AsMe_3$ < I^- < OH^- < Br^- < Cl^- < SMe_2 < pyridine < D_2O. They concluded that the spin-spin coupling constant gives a measure of the metal-ligand bond strength, or at least the s-orbital contribution to it. The spin-spin coupling constant has been observed to depend linearly on the relative reactivities of X^- in S_N2 type reactions and has been interpreted to indicate that the affinity of various X groups for the methylmercury cation is linearly dependent on the acidity of the respective acids HX (38).

The results described above indicate that both the chemical shift of the methyl protons and the mercury-proton spin-spin coupling constant depend on the nature of the metal-ligand bond. Because of this dependence, it would seem that the donor group to which methylmercury is complexed in molecules such as amino acids and peptides containing more than one known potential binding site could possibly be identified from the magnitude of these parameters for the complexed methylmercury. In addition, the potential binding sites of the ligand can be monitored by means of the NMR spectra of the ligand nuclei. In Chapter IV, the nature of the methylmercury binding by selected amino acids and the peptide glutathione is

characterized by nuclear magnetic resonance spectroscopy and, where possible, formation constants for the complexation have been determined. In Chapter V, the results of NMR studies of the dynamics of the reactions of methylmercury with several of these molecules are presented.

CHAPTER II

EXPERIMENTAL

A. Chemicals

S-methyl-L-cysteine (Nutritional Biochemicals Corp.), D,L-methionine (British Drug Houses), D,L-penicillamine(Aldrich Chemical Comp.), D,L-valine, tetramethylammonium (TMA) hydroxide (Eastman Organic Chemicals), tertiary-butanol, methylamine, 1,4-dioxane (Fisher Scientific Company) were used as received. L-cysteine (Nutritional Biochemicals Corp.) was recrystallized from water as the free base. Reduced glutathione (Nutritional Biochemicals Corp., Terochem Laboratories, and Sigma Chemical Company) was washed with a water-ethanol solution and dried at 110°C before using. Methylmercuric hydroxide (Alfa Inorganics) was purified as described below.

All other chemicals were reagent grade and were used without further purification.

B. Preparation and Standardization of Methylmercuric Hydroxide

The commercial methylmercuric hydroxide contained an acetate impurity (40), as indicated by a singlet in the proton magnetic resonance spectrum at 1.91 ppm downfield from the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5 sulfamic acid (DDS), and an insoluble material.

The methylmercuric hydroxide was purified by first passing an approximately 0.4 M solution in triply-distilled water through a 0.2 micron membrane filter two or three times to remove the insoluble fraction. The filtrate was then passed through an anion exchange column (DOWEX 1-X8) in the hydroxide form to remove the acetate ions. Since sodium hydroxide would not interfere in the studies of methylmercury binding no attempt was made to remove the sodium hydroxide.

The solution was then analyzed. The total hydroxide concentration of the solution was determined by adding a small excess of sodium thiosulphate to displace the hydroxide from the methylmercury hydroxide

$$CH_3HgOH + S_2O_3^{=} \rightleftharpoons CH_3HgS_2O_3^{-} + OH^{-} \qquad (6)$$

The hydroxide was then titrated with standard nitric acid and the end point was located potentiometrically by measuring the pH as acid was added. The total hydroxide concentration of a typical stock solution which would be equal to the methylmercury concentration only if no other cation was present was found to be 0.366 ± 0.005 M. By flame photometry it was found that this stock solution contained 0.030 ± 0.005 M sodium ion while, by the NMR titration procedure described below, the methylmercury concentration of the same solution was determined to be 0.34 ± 0.01. These results indicate that, before being

passed through the ion exchange column, the solution consists of methylmercuric hydroxide and sodium acetate. On passing through the ion exchange column the sodium acetate is converted to sodium hydroxide.

Ion-exchanged solutions of methylmercuric hydroxide were used as stock solutions and were standardized by one of the following two methods. The first of these methods is an NMR titrimetric method based on the formation of a 1:1 complex from methylmercuric hydroxide and thiosulphate as described by Equation (6). The titration was performed in an NMR tube with the titrant being added gravimetrically from a syringe. The endpoint in the titration was determined by PMR by utilizing the change in the chemical shift of the methylmercury protons as titrant is added. On the PMR time scale, exchange of $CH_3Hg(II)$ between OH^- and $S_2O_3^=$ is rapid resulting in a single averaged resonance pattern whose chemical shift is an average of these parameters for the two complexes, weighted according to their relative concentrations. As thiosulphate titrant is added, the chemical shift changes linearly from that of CH_3HgOH to that of $CH_3HgS_2O_3^-$. The endpoint was determined by extrapolation of the straight line portions of a plot of chemical shifts vs. grams thiosulfate added. From the grams of thiosulfate solution required to reach the endpoint and the densities of the solutions, the concentration of methylmercuric hydroxide in the stock solution was calculated.

Because the NMR titration procedure is time consuming and lacks precision, a faster and more precise potentiometric method based on the reaction of chloride ions with methylmercury to form methylmercuric chloride was developed.

$$CH_3HgOH_2^+ + Cl^- \rightleftharpoons CH_3HgCl \qquad (7)$$

$$K_F = \frac{[CH_3HgCl]}{[CH_3HgOH_2^+][Cl^-]} \qquad (8)$$

The formation constant for methylmercuric chloride in aqueous solution is 1.78×10^5 (2). The endpoint was determined potentiometrically using a silver/silver chloride indicating electrode and a saturated calomel reference electrode. To minimize the competition of hydroxide with titrant for $CH_3Hg(II)$, the titration was performed in acidic solution where the major fraction of methylmercury exists as the hydrated cation. Initially the titration was attempted at pH 2 in aqueous solution, yielding the titration curve shown in Figure 1. The titration curve for these conditions is drawn out, making it difficult to obtain precise endpoints. To increase the formation constant and thus the sharpness of the break at the endpoint, the titration was performed in a mixed water-ethanol solvent system. Titration curves for a range of solvent conditions are shown in Figure 2. In the 80%

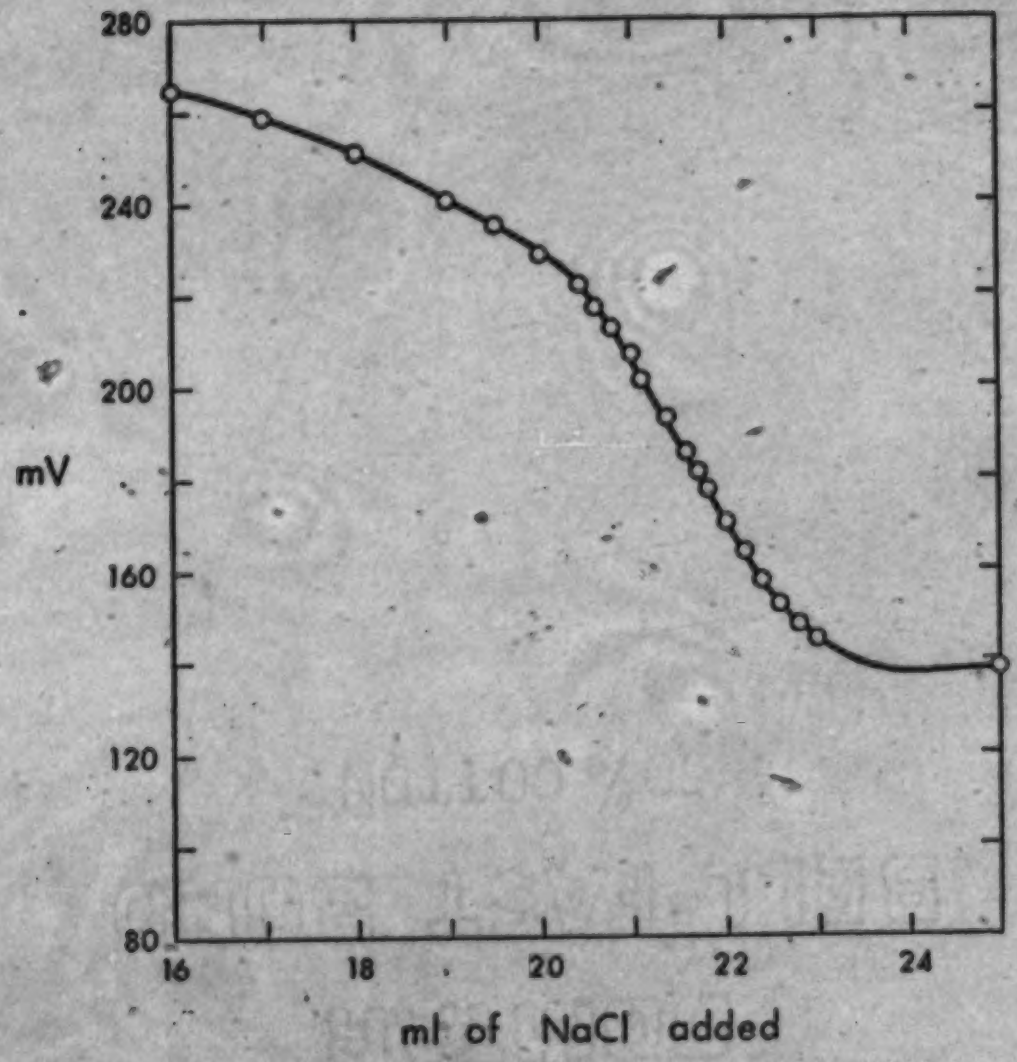


Figure 1: Potentiometric titration curve for 5.00 ml of methylmercury stock solution diluted to 100 ml with distilled water. The pH was adjusted to 2 with concentrated nitric acid. The titrant was 0.1024 M sodium chloride.

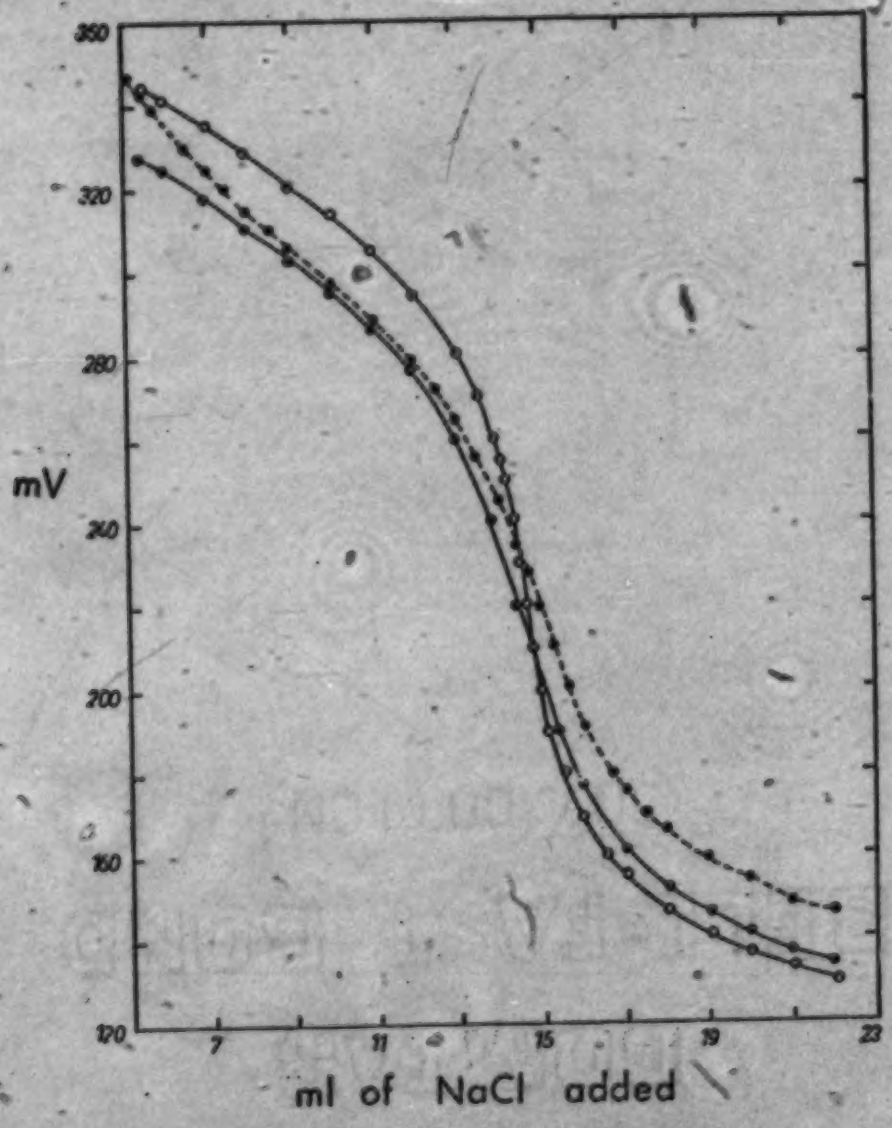


Figure 2: Potentiometric titration curves for 5.00 ml of methylmercury solution diluted to 105 ml with distilled water (- ● -), 20% ethanol (- ○ -) or 80% ethanol (- ◔ -). The pH was adjusted to 2 with concentrated nitric acid. The titrant was 0.0300 M sodium chloride.

ethanol by volume titration the potential changed by 0.194 v. on going from 1% before to 1% after the endpoint. The 80% ethanol solvent system was adopted as titration medium. The analysis procedure involved pipetting 5 ml of methylmercuric hydroxide stock solution into a 150 ml beaker and adding to it 15 mls of 0.3 M nitric acid to reduce the pH in the final solution to approximately 2. 80 mls of 95% ethanol were then added and the solution titrated with standard sodium chloride. The concentration of a typical stock solution was found after 4 titrations to be 0.4463 ± 0.0005 M methylmercury.

C. Preparation of Other Solutions

A stock solution of tetramethylammonium nitrate, used as a reference for some of the chemical shift measurements, was prepared by titration of a 25% aqueous solution of TMA hydroxide with nitric acid to a neutral pH.

The solutions used in the proton magnetic resonance (PMR) and carbon-13 magnetic resonance (CMR) measurements were prepared in triply-distilled water from pipetted amounts of stock methylmercury solution and weighed or pipetted amounts of the ligand. TMA nitrate, t-butanol, or dioxane was added for an internal chemical shift reference at approximate concentrations of 0.01, 0.03, and 0.07 M respectively. To avoid dilution, the pH was adjusted with concentrated nitric acid or a concentrated

solution of potassium hydroxide and samples of about 0.4 ml for PMR or 2.5 ml for CMR were withdrawn at the desired pH values. When mole ratio measurements were being performed at constant pH, the requisite amount of ligand or methylmercury was added, the pH adjusted and a sample withdrawn. More ligand or methylmercury was added to the solution and the same procedure followed for all samples at this pH. The concentrations were corrected for the changing solution volume. Since high concentrations are necessary in the PMR and CMR experiments (greater than 0.1 M), no attempt was made to control the ionic strength.

When standard nitric acid was required it was prepared from concentrated acid and standardized with primary standard mercuric oxide (39). The procedure involved weighing accurately samples of dry mercuric oxide into Erlenmeyer flasks and then adding approximately 5 moles of potassium iodide dissolved in a small volume of water for each mole of mercuric oxide. The iodide reacts with the mercuric oxide to form two moles of hydroxide for each mole of mercuric oxide according to the following displacement reaction.

$$HgO + 4I^- + H_2O \rightleftarrows HgI_4^= + 2OH^- \qquad (9)$$

The nitric acid solution was standardized by titration of the hydroxide to a phenolphthalein endpoint. Standard solutions of sodium hydroxide were prepared from saturated

carbonate-free sodium hydroxide solutions, and standardized by titration with standardized nitric acid. A stock solution of methylamine was prepared from a weighed amount of 40% aqueous solution and standardized by titration with standardized nitric acid.

D. pH Measurements

pH measurements were made at 25 ± 1°C with an Orion Model 801 pH meter or a Fisher Model 520 pH meter equipped with either a standard glass electrode and a fibre tip junction saturated calomel reference electrode or a micro-combination electrode. Saturated potassium acid tartrate, 0.050 M phosphate, and 0.01 M sodium tetraborate solutions, pH values 3.56, 7.00 and 9.18 at 25°C were used to standardize the pH meter.

E. Proton Magnetic Resonance Measurements

Proton magnetic resonance spectra were obtained at 60 MHz on a Varian A-60-D spectrometer and at 100 MHz on a Varian HA-100 spectrometer. Both instruments were equipped with Varian variable temperature controllers. At temperatures less than 25°C, the temperature of the probe was determined using the chemical shift difference between the methyl and hydroxyl resonances of a standard methanol sample and the appropriate equation of the following set (40).

-98° - -48°	$T = 264.4 - 2.380\|\Delta\delta\|$	(10)
-53° - -3°	$T = 225.4 - 2.083\|\Delta\delta\|$	(11)
- 8° - -40°	$T = 195.0 - 1.810\|\Delta\delta\|$	(12)

where $|\Delta\delta|$ is the absolute value in Hz of the chemical shift difference. At temperatures greater than 25°C, an ethylene glycol sample was used with the calibration plot of chemical shift difference between methyl and hydroxyl resonances versus temperature supplied by Varian.

Spectra were recorded at sweep rates of 0.1 Hz/sec for the chemical shift measurements and 0.5 Hz/sec for the spin-spin coupling measurements. Reported data are the average of several scans. Chemical shifts were measured relative to the central resonance of the TMA triplet or the t-butyl resonance of t-butanol. For all measurements, the chemical shifts are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) unless otherwise noted. Positive shifts correspond to resonances of protons less shielded than those of the reference. The t-butyl resonance of t-butanol is 1.24 ppm downfield from the methyl resonance of DSS while the central resonance of TMA is 3.17 ppm downfield from the methyl resonance of DSS.

F. Carbon-13 Magnetic Resonance Measurements

Carbon-13 magnetic resonance spectra were obtained using a Bruker HFX-90 spectrometer operating at a

frequency of 22.63 MHz and equipped with a Nicolet 1085 computer. The Fourier transform mode was used with proton decoupling. For each free induction decay signal, 8K data points were collected in the computer. The spectra were time averaged using 4k or 8k accumulations. The fluorine-19 resonance from C_6F_6 in a coaxial capillary was used for the lock. The frequency range of the transformed spectra was 5000 Hz. Chemical shifts are reported in ppm relative to the carbon-13 resonance of 1,4-dioxane added as an internal reference. The resonance of 1,4-dioxane is 67.4 ppm downfield from the TMS resonance. Positive chemical shifts correspond to less shielding than in 1,4-dioxane. The chemical shift measurements are considered accurate to within 0.05 ppm. For all measurements the sample temperature was 25 ± 1°C as measured with a standard thermocouple.

G. Determination of Formation Constants by NMR Spectroscopy

In Chapter IV of this thesis, formation constants for methylmercury complexation reactions, defined by Equations (13) and (14), have

$$CH_3HgOH_2^+ + L \rightleftarrows CH_3HgL + H_2O \qquad (13)$$

$$K_F = \frac{[CH_3HgL]}{[CH_3HgOH_2^+][L]} \qquad (14)$$

been determined by NMR spectroscopy. The NMR spectrum of methylmercury and/or the ligand reflects the complexation, with the exact nature of the effect on the spectrum being dependent on the rate of exchange of the species being observed between its free and complexed forms. If the rate of exchange is slow, that is the reciprocal of the lifetime of the free and complexed forms is less than $2\pi\Delta\delta$, where $\Delta\delta$ represents the separation in Hz of the resonances of the free and complexed forms, then separate resonances are observed for the free and complexed forms of methylmercury and the ligand. The intensity of each resonance is proportional to the concentration of that form from which the formation constant can be calculated directly.

If the rate of exchange is fast, that is the inverse of the lifetime is somewhat greater than $2\pi\Delta\delta$, then exchange-averaged spectra are observed for both the methylmercury and ligand resonances. The observed shift is a weighted average of the two forms as described by

$$\delta_{OBS} = P_f\delta_f + P_c\delta_c \qquad (15)$$

where δ_{OBS} represents the observed shift, δ_c represents the shift of the complexed ligand or methylmercury, and δ_f represents the shift of the free ligand or methylmercury. P_f and P_c are the fractional concentrations of the free and complexed forms. Substituting $P_f + P_c = 1$ into

Equation (15), one obtains

$$P_c = \frac{\delta_{OBS} - \delta_f}{\delta_c - \delta_f} \qquad (16)$$

The fractional concentrations P_c and P_f can be obtained from the observed shift and the shifts of the free and complexed forms, and the formation constant can be calculated from P_c and P_f and the total concentration of ligand.

To determine formation constants by the method described above, it is necessary that solution conditions be such that there is a significant fractional concentration of each form so that δ_{OBS} differs from δ_f and δ_c. This can be accomplished by utilizing competing equilibria. In acidic solution, competition between protons and methylmercury for the ligand can be utilized while in basic solutions the ligand and hydroxyl ions are competing for methylmercury. Using the fractional concentration determined for the particular solution conditions from the observed chemical shift and the equilibrium constant for the competing reaction, the formation constant can then be calculated.

H. Kinetic Applications of NMR Spectroscopy

The basic theories used in the study of chemical exchange reactions by nuclear magnetic resonance have been

treated extensively in several monographs and review articles (41-45). The modified Bloch approach has been utilized in the present work to extract kinetic data from PMR and CMR spectra. Only a brief qualitative description of this approach will be given.

Bloch (46) obtained a set of equations to describe the time dependence of the components of the total nuclear magnetic moment vector per unit volume for a collection of nuclei with non-zero spins in the presence of a non-stationary magnetic field. The simplest chemical system in which chemical exchange may be studied by NMR is one in which the nuclei are interchanging between two different chemical environments, f and c, with different Larmor frequencies (chemical shifts). For this case, the time dependence of the magnetization vectors from nuclei in environments f and c can be described by Bloch equations. Addition of terms to these equations to account for the transfer of magnetization between the two sites leads to the modified Bloch equations (47). Solution of the coupled equations for the two site exchange leads to a general expression (Equation 17) for the intensity of the NMR absorption at a given frequency, ω (in radians/sec), when there is exchange between the sites (48,49). In Equation (17), M_y represents the absorption signal, $\Delta\omega_c - \Delta\omega_f$ represents the chemical shift difference of the nucleus in the two environments, $1/T_{2c}$ and $1/T_{2f}$ are the magnetic re-

$$M_y \alpha \frac{\{[P_c\Delta\omega_f + P_f\Delta\omega_c][\Delta\omega_c(\frac{1}{T_{2f}} + \frac{1}{\tau_f}) + \Delta\omega_f(\frac{1}{T_{2c}} + \frac{1}{\tau_c})] + [(\frac{P_f}{T_{2c}} + \frac{1}{\tau_c}) + (\frac{P_c}{T_{2c}} + \frac{1}{\tau_f})][(\frac{1}{T_{2c}} + \frac{1}{\tau_c})(\frac{1}{T_{2f}} + \frac{1}{\tau_f}) - \Delta\omega_c\Delta\omega_f - \frac{1}{\tau_c}\frac{1}{\tau_f}]\}}{[(\frac{1}{T_{2c}} + \frac{1}{\tau_c})(\frac{1}{T_{2f}} + \frac{1}{\tau_f}) - \frac{1}{\tau_c}\frac{1}{\tau_f} - \Delta\omega_f\Delta\omega_c]^2 + [\Delta\omega_c(\frac{1}{T_{2f}} + \frac{1}{\tau_f}) + \Delta\omega_f(\frac{1}{T_{2c}} + \frac{1}{\tau_c})]^2} \quad (17)$$

laxation times (line widths) in the absence of chemical exchange, and τ_c and τ_f represent the average time the nucleus spends at site c or f before an exchange occurs. This general equation may be simplified when the rate of chemical exchange satisfies certain limiting conditions.

When the rate of chemical exchange is slow, the spectrum consists of two separate resonances at frequencies δ_f and δ_c (in Hz). For this situation the average lifetime τ_f and τ_c of the nuclei in each of the sites before exchange to the other site are large compared to the inverse of the separation of the resonances; that is, τ_f or τ_c is greater than $2\pi(\delta_c - \delta_f)^{-1}$. These lifetimes may be determined from the extent of broadening of each of the resonances by (42)

$$\frac{1}{\tau_c} = \pi(W'_{1/2,c} - W_{1/2,c}) \quad (18)$$

where $W'_{1/2,c}$ is the width at half height of the exchange-broadened resonance of nuclei in the complexed site and $W_{1/2,c}$ is the width of this resonance in the absence of exchange. An analogous equation may be written for the lifetime τ_f. The linewidth $W_{1/2,c}$ is related to the effective transverse relaxation time T_{2c} by the relation $\frac{1}{T_{2c}} = \pi W_{1/2,c}$, which takes into account the actual spin-spin relaxation time and broadening due to magnetic field inhomogeneities.

For the condition of intermediate exchange, the lifetimes are of the order of the inverse of the separation of the resonances and the spectrum consists of partially-coalesced resonances. When the lifetimes are small compared to the inverse of the separation of the resonances, exchange is rapid and the spectrum consists of an averaged resonance at a frequency which is the population weighted average of δ_f and δ_c. [Equation (15)]. Under fast exchange conditions, the inverse of the lifetime of the complexed species, $1/\tau_c$, is related to the various spectral parameters and the fractional concentrations by (50)

$$\frac{1}{\tau_c} = \frac{P_f^2 P_c 4\pi(\delta_f - \delta_c)^2}{W_{1/2,OBS} - (P_f W_{1/w,b}) - (P_c W_{1/w,c})} \qquad (19)$$

where δ_f and δ_c represent the shifts of the free and complexed forms (in Hz), $W_{1/2,OBS}$ is the observed linewidth,

$W_{1/2,f}$ is the linewidth of the free form and $W_{1/2,c}$ is the linewidth of the complexed form.

For the methylamine system discussed in Chapter V exchange between free and complexed forms is rapid on the NMR timescale and lifetimes were calculated using Equation (I9). For the glutathione system also discussed in Chapter V, the lifetimes in the individual sites were obtained by computing theoretical spectra as a function of lifetime using modified Bloch equations and then matching them to experimental spectra.

CHAPTER III

METHYLMERCURY SPECIES AND EQUILIBRIA IN AQUEOUS SOLUTION

A. Introduction

The aqueous solution model used for methylmercury chemistry in the majority of studies of the aqueous solution chemistry of methylmercury complexes is that of Schwarzenbach and Schellenberg (2). This model was developed from pH titration data for solutions containing 5.85×10^{-4} M to 2.19×10^{-2} M methylmercury. In the Schwarzenbach and Schellenberg model, $CH_3HgOH_2^+$ reacts with hydroxide ion to form CH_3HgOH which further reacts with CH_3HgOH to form $(CH_3Hg)_2OH^+$ according to Equations (20) - (24)

$$CH_3HgOH_2^+ + OH^- \rightleftharpoons CH_3HgOH + H_2O \qquad (20)$$

$$K_1 = \frac{[CH_3HgOH]}{[CH_3HgOH_2^+][OH^-]} \qquad (21)$$

$$CH_3HgOH_2^+ + CH_3HgOH \rightleftharpoons (CH_3Hg)_2OH^+ + H_2O \qquad (22)$$

$$K_2 = \frac{[(CH_3Hg)_2OH^+]}{[CH_3HgOH_2^+][CH_3HgOH]} \qquad (23)$$

Woodward and coworkers (51-53) identified the species CH_3HgOH in aqueous solution by Raman spectroscopy and assigned the band at 415 cm^{-1} in the Raman spectra to

$(CH_3Hg)_2OH^+$. This assignment of the Raman spectrum to CH_3HgOH has been criticized by Green (16) on the basis of a report by Grdenic and Zado (17) that CH_3HgOH does not exist as a chemical compound except possibly in dilute aqueous solution as the dissociation product of trismethylmercurioxonium hydroxide, $[(CH_3Hg)_3O]OH$, which they claimed to have prepared by a metathetical reaction between $[(CH_3Hg)_3O]ClO_4$ and KOH in methanol. They concluded from conductance data that $(CH_3Hg)_3O^+$ is stable in alkaline (methanol) solution and thus that the compound previously thought to be CH_3HgOH is actually $[(CH_3Hg)_3O]OH$ or its dehydration product. Lorbeth and Weller (54) have shown that the compound reported to be $[(CH_3Hg)_3O]OH$ by Grdenic and Zado is a hydrate of $(CH_3Hg)_2O$. Grdenic and Zado (17) also observed that a small amount of the trismethylmercurioxonium perchlorate, $[(CH_3Hg)_3O^+]ClO_4^-$, precipitated when a concentrated aqueous solution of methylmercuric hydroxide is titrated with perchloric acid, indicating that some trismethylmercurioxonium ion must be present in aqueous solution. If so an additional reaction must be added to the Schwarzenbach and Schellenberg model to include formation of the species, $(CH_3Hg)_3O^+$.

$$CH_3HgOH + (CH_3Hg)_2OH^+ \rightleftarrows (CH_3Hg)_3O^+ + H_2O \quad (24)$$

$$K_3 = \frac{[(CH_3Hg)_3O^+]}{[CH_3HgOH][(CH_3Hg)_2OH^+]} \quad (25)$$

Goggin and Woodward (53) determined by Raman spectroscopy that methylmercuric perchlorate is completely dissociated into $CH_3HgOH_2^+$ and ClO_4^- in aqueous solution as predicted by the Schwarzenbach and Schellenberg model. They also found that, in benzene, methylmercuric nitrate is not dissociated whereas in 4 M aqueous solution there is an equilibrium between undissociated solute and the ions, $CH_3HgOH_2^+$ and NO_3^-. The degree of dissociation was found to be similar to that of nitric acid in aqueous solution. Clarke and Woodward (55) found by Raman spectroscopy that the complex of methylmercury with methanesulfonate ions is partially dissociated in 4.7 M aqueous solutions and completely dissociated in 1 M solution. In the methanesulfonate complex of methylmercury, the methanesulfonate is characterized by monodentate bonding through an oxygen. The same workers concluded that methylmercury forms a 1:1 complex with sulfate, $CH_3HgSO_4^-$, which is dissociated to approximately the same extent as HSO_4^- in aqueous solution.

In this chapter, NMR and Raman results are reported for the aqueous solution chemistry of methylmercury. These results indicate that the Schwarzenbach and Schellenberg model accounts for most of the methylmercury species except at high methylmercury concentrations where some trismethylmercurioxonium ion forms in the neutral pH region. This research was done in collaboration

with M.C. Tourangeau and Dr. C.A. Evans (56).

B. Results and Discussion

The PMR spectrum of the methyl group of methylmercuric hydroxide in aqueous solution at pH 12.0 is shown in Figure 3. The spectrum consists of a singlet symmetrically flanked by two less intense lines. The central resonance is due to methyl groups bonded to isotopes of mercury having a nuclear spin of zero while the satellites are due to methyl groups bonded to mercury - 199 (16% natural abundance, I = 1/2). The chemical shift of the methyl protons is given by the shift of the central resonance relative to the central resonance of TMA and the mercury-proton coupling constant is given by the separation between the satellite lines. The chemical shift of the methyl resonance and the mercury-proton coupling constant for a methylmercury solution containing no coordinating ligand other than hydroxide are pH dependent. The chemical shift as a function of pH is presented in Figure 4 and the coupling constant as a function of pH is given in Figure 5. Both are for a solution containing 0.190 M methylmercury. If the model represented by Equations (20) - (25) describes the aqueous solution chemistry of methylmercury, th fractional concentrations of $(CH_3Hg)_2OH^+$ and $(CH_3Hg)_3O^+$ at a given pH will be dependent on the total methylmercury concentration due to the unsymmetrical nature of the

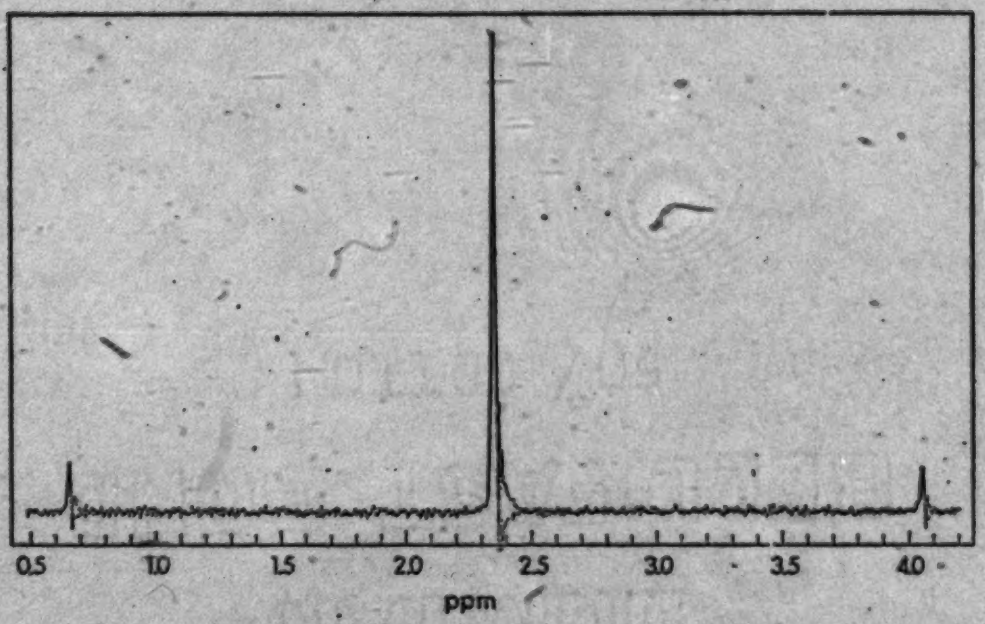


Figure 3: PMR spectrum of the methyl group of methylmercury in a 0.190 M aqueous solution at pH = 12.0.

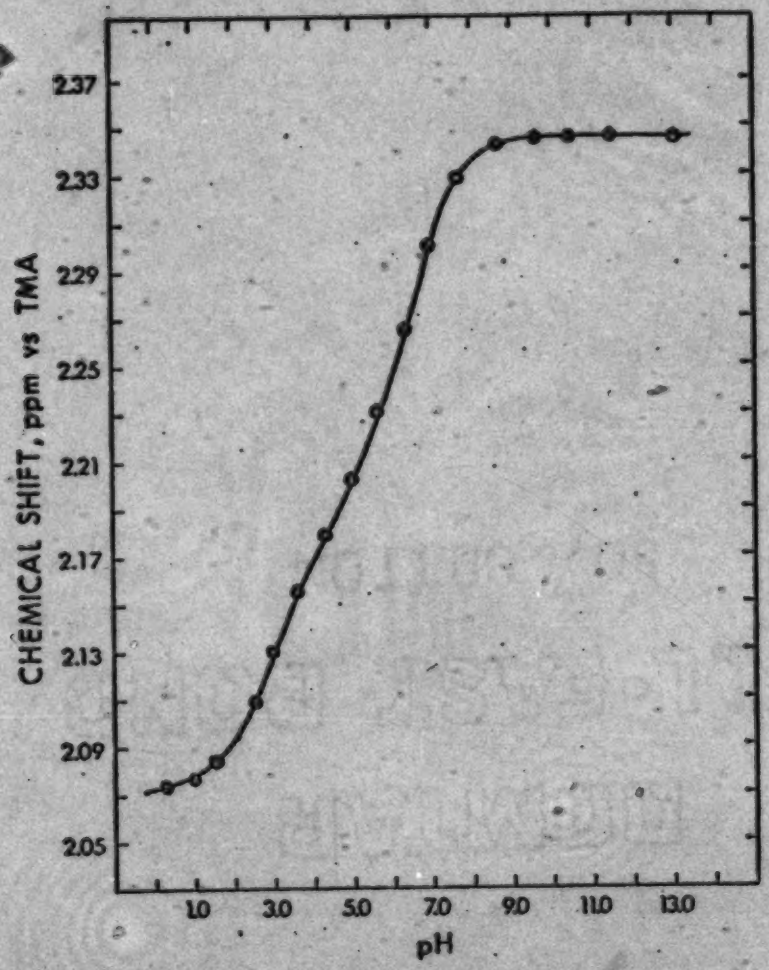


Figure 4: pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 M methylmercury. The curves connecting the points are theoretical curves calculated using the constants given in the thesis.

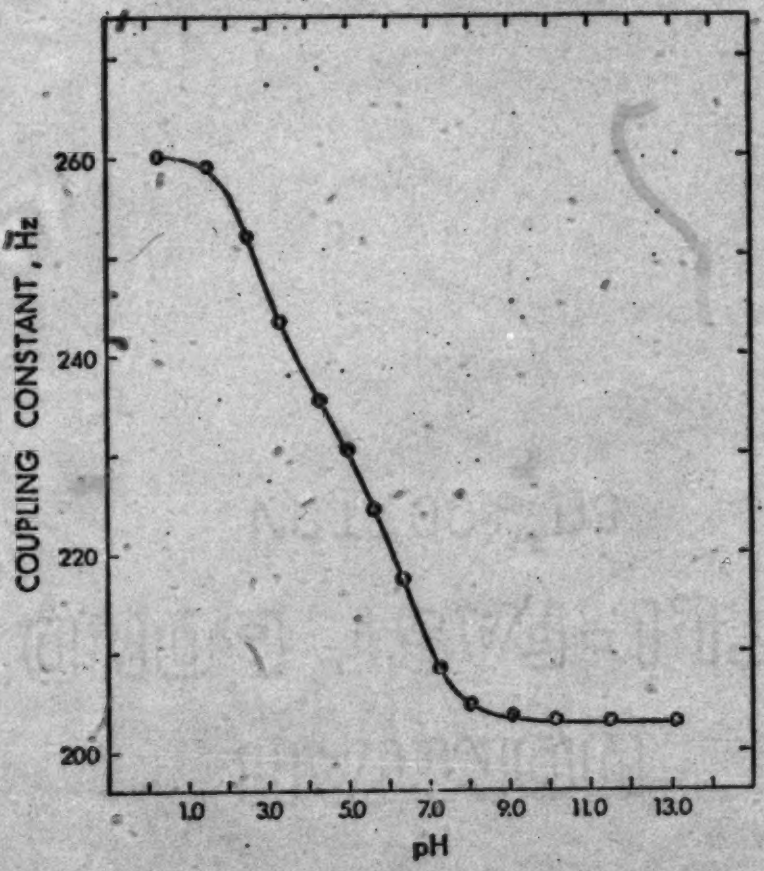


Figure 5: pH dependence of the mercury-proton spin-spin coupling constant of methylmercury in an aqueous solution containing 0.190 M methylmercury. The curves connecting the points are the theoretical curves calculated using the constants given in the thesis.

equilibria for their formation. Also the species distribution will be pH dependent, with CH_3HgOH the predominant species above a certain pH. The chemical shift of the protons of the methyl group and the ^1H-^{199}Hg coupling constant change by 0.273 ppm and 57.0 Hz. respectively as the pH is increased from 0.26 to ~9 and then remain constant as the pH is increased further. Other measurements have shown that, at pH greater than 9, the chemical shift is the same for total methylmercury concentrations ranging from 0.00437 to 0.45 M (56). Also $[(CH_3Hg)_3O]ClO_4$ does not precipitate from a pH 12 solution containing 0.43 M methylmercury and 0.49 M $NaClO_4$, indicating that the concentration of $(CH_3Hg)_3O^+$ is less in basic solution than at neutral pH. These results indicate that CH_3HgOH is the predominant species at pH greater than 9 at these and lower total methylmercury concentrations.

The non-sigmoid nature of the chemical shift titration curve in Fig. 4 indicates that species in addition to CH_3HgOH and $CH_3HgOH_2^+$ are present in the pH region 1 to 8. It is not possible to observe the different species directly by NMR since time-averaged spectra are observed for rapidly exchanging systems such as methylmercury. However, Raman spectroscopy is capable of establishing the presence of possible methylmercury species in solution if bands specific to the individual species are observable.

Raman and infrared spectra of crystalline $[(CH_3Hg)_3O]ClO_4$ were recorded to determine the bands characteristic of $(CH_3Hg)_3O^+$ (56). Except for solvent bands, the Raman spectra of methanol and nitromethane solutions of $[(CH_3Hg)_3O]ClO_4$ are similar to those of the solid, which indicates that the cation is undissociated in these solvents. Clarke and Woodward (57) also found that $(CH_3Hg)_3O^+$ is undissociated in acetonitrile. However, the spectrum of a saturated aqueous solution of $[(CH_3Hg)_3O]ClO_4$ shows only one intense band in the region 500 - 600 cm^{-1}, at 568 cm^{-1}. In addition, some weak to medium intensity bands appear in the region 400 - 500 cm^{-1}. These changes can be interpreted in terms of hydrolysis of the cation.

Grdenic and Zado (17) had concluded from conductimetric titrations of methanolic solutions of tris(methylmercuric)oxonium salts with methanolic KOH that $(CH_3Hg)_3O^+$ is undissociated in alkaline methanol solution. To determine if this is the case, the chemical shift and mercury-proton coupling constant were measured as methanol solutions prepared from $[(CH_3Hg)_3O]ClO_4$ were titrated with methanolic KOH. Similar experiments were also performed in aqueous solution. The chemical shift results, which are presented in Figure 6, indicate that $(CH_3Hg)_3O^+$ is not stable in either alkaline methanol or alkaline aqueous solution. The Raman results described above and the stoichiometry

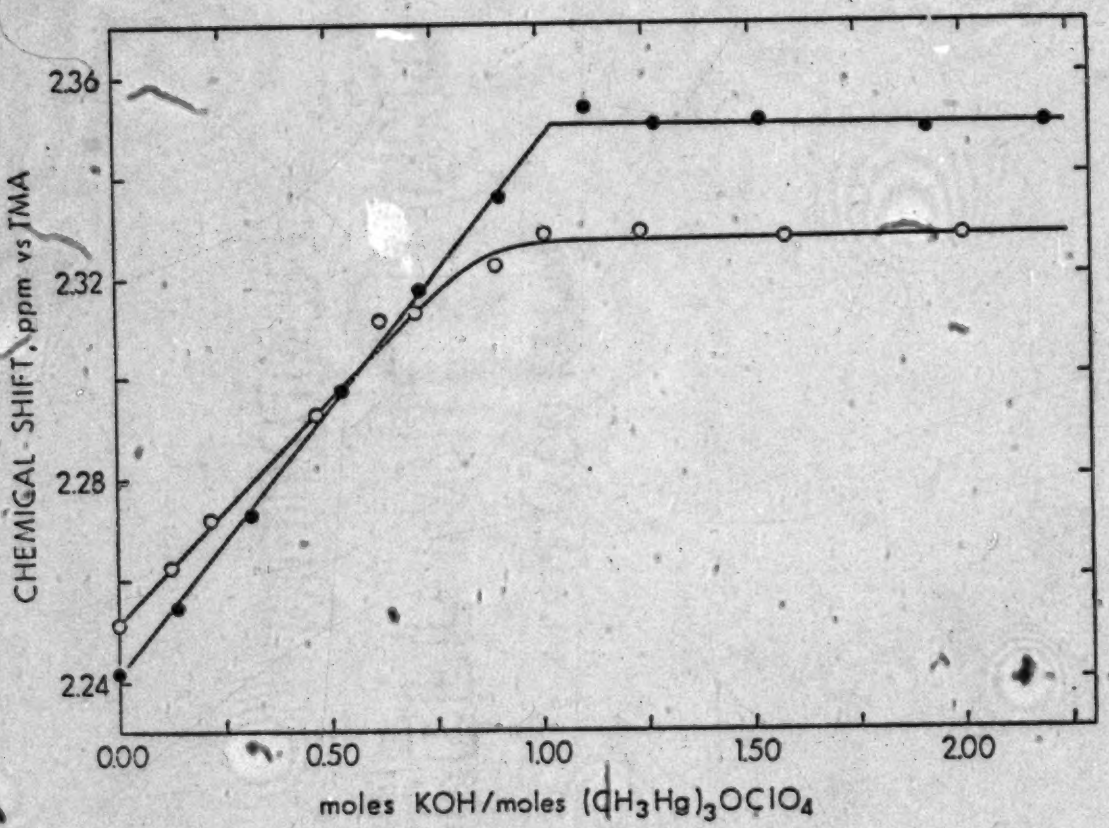


Figure 6: Solid points: NMR titration curve for the titration of 0.76 g of $[(CH_3Hg)_3O]ClO_4$ in 80 ml of water with 1.0 M aqueous KOH. Open points: NMR titration of 0.76 g of $[(CH_3Hg)_3O]ClO_4$ in 80 ml of methanol with 1.0 M methanolic KOH.

of the titration suggest that, in aqueous solution, $(CH_3Hg)_3O^+$ hydrolyzes to form CH_3HgOH and $(CH_3Hg)_2OH^+$. When base is added, the $(CH_3Hg)_2OH^+$ then reacts to form CH_3HgOH. In methanol, undissociated $(CH_3Hg)_3O^+$ reacts with hydroxide, probably to form CH_3HgOH and $(CH_3Hg)_2O$. These results are consistent with the report by Lorbeth and Weller (54) that the substance claimed to be $[(CH_3Hg)_3O]OH$ is actually the hydrated oxide $(CH_3Hg)_2O \cdot xH_2O$

The pH dependence of the species distribution has been determined by Raman spectroscopy by Dr. C.A. Evans. Raman spectra of a 0.510 M solution titrated with 5.68 M perchloric acid were recorded over the pH range 9 to 0.5 at intervals of approximately 0.5 pH unit (56). The high concentration was chosen to favour the formation of $(CH_3Hg)_3O^+$. In two of the samples, at pH 6.84 and 6.26, crystals of $[(CH_3Hg)_3O]ClO_4$ slowly appeared. From various features in the Raman spectrum of the super-saturated solutions and the crystals at these pH's, the $(CH_3Hg)_3O^+$ cation does not appear to be a major species in aqueous solution, even when the solution is super-saturated with respect to $[(CH_3Hg)_3O]ClO_4$ (56). From the intensity of the band at 504 cm^{-1}, which was assigned to methylmercuric hydroxide, K_3 was estimated to be 0.7 with a probable deviation of 0.3. Although this value of K_3 lacks precision, it is expected to be of the correct order of magnitude and leads to some important conclusions.

Calculations of species distributions, based upon this value for K_3, as a function of pH indicate that, for a 0.5I M methylmercury solution, the maximum concentration of $(CH_3Hg)_3O^+$ is 0.019 M and this occurs at a pH of 6.75, while, at methylmercury concentrations of 0.2 and 0.05, the maximum concentrations are 3.1×10^{-3} M (pH 6.4) and 2.0×10^{-4} M (pH 5.8). The pH dependence of the fractional concentrations of the various species in a 0.2 M methylmercury solution is shown in Figure 7. These results are based on the model described by Equations (20) - (25) with log K_1 = 2.31, log K_2 = 9.29, and K_3 = 0.7. The Schwarzenbach and Schellenberg model accounts for all but a small fraction of the methylmercury over the pH range less than 1 to greater than 13 at the methylmercury concentrations which have been used in most of the previous studies of the solution chemistry of methylmercury complexes (15) and those described in Chapters IV and V of this thesis.

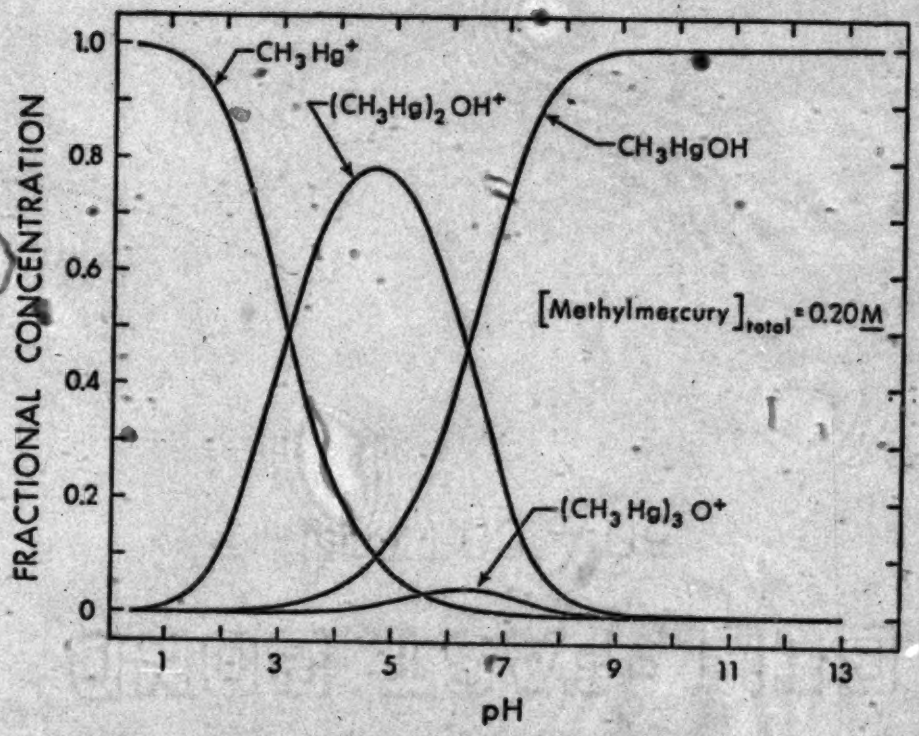


Figure 7: Fractional concentrations of the methylmercury-containing species in an aqueous solution containing 0.200 M methylmercury. The fractional concentrations were calculated using constants given in the thesis.

CHAPTER IV

The Binding of Methylmercury by Selected Amino Acids and by the Peptide Glutathione

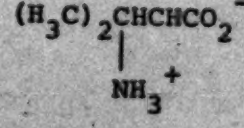
A. Introduction

To characterize the binding of methylmercury by the potential methylmercury coordination sites in peptides and proteins, the binding of methylmercury by the amino acids valine, methionine, cysteine, and penicillamine and the tripeptide glutathione was investigated. Valine was chosen as a model compound to investigate binding by carboxyl and amine groups; methionine, to characterize the interaction with the thioether group; and penicillamine, cysteine and glutathione, because of the known affinity of methylmercury for the sulfhydryl group. The results of NMR studies of the complexation of methylmercury by these ligands in aqueous solution are presented in this chapter (58-60).

B. Results

1. The Binding of Methylmercury by Valine

The structural formula of valine in the zwitterion form is given by I. The binding of methylmercury by valine was investigated using the chemical shift of



I

the methyl protons and the mercury-proton coupling constant of methylmercury. The chemical shift is presented as a function of pH for both a solution containing equal concentrations of methylmercury and valine (open points) and a solution of methylmercury alone (solid points) in Figure 8. The coupling constant data for the same solutions is presented in Figure 9. As shown in the figures, the two titration curves approach each other at both very low and very high pH. At very low pH, the high concentration of hydrogen ions results in protonation of the ligand and displacement of the methylmercury, while at very high pH hydroxyl ions compete with the ligand for the methylmercury. Thus complexation is favored in the intermediate pH region.

In valine there are two potential coordination sites for methylmercury: the deprotonated amino and carboxyl groups. The acid dissociation constants for these groups defined by Equations (26) - (29) are $pK_{A1} = 2.29$ and $pK_{A2} = 9.81$ (61)

$$(CH_3)_2CHCH(NH_3^+)COOH + H_2O \rightleftarrows (CH_3)_2CHCH(NH_3^+)COO^- + H_3O^+ \quad (26)$$

$$K_{A1} = \frac{[HL^{+-}][H_3O^+]}{[H_2L^+]} \quad (27)$$

$$(CH_3)_2CHCH(NH_3^+)COO^- + H_2O \rightleftarrows (CH_3)_2CHCH(NH_2)COO^- + H_3O^+ \quad (28)$$

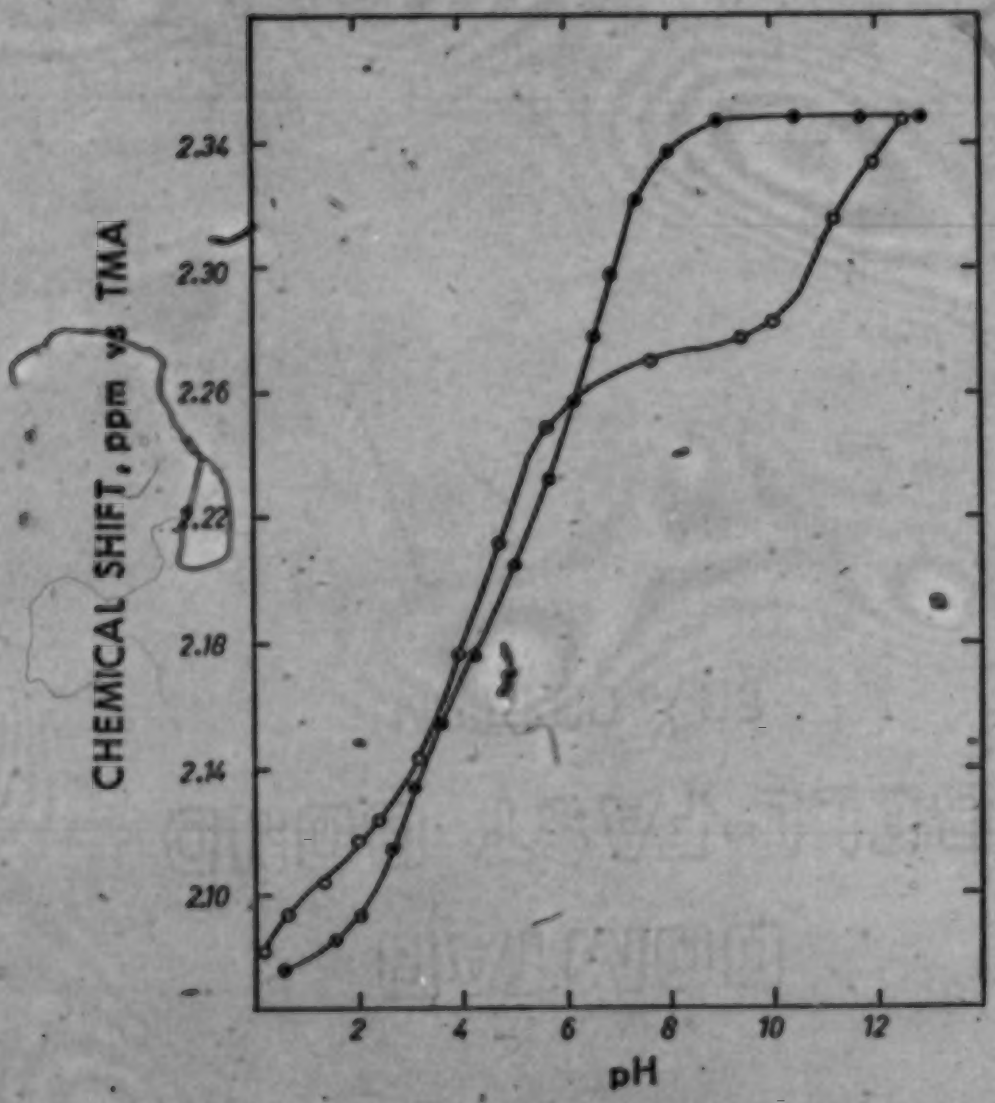


Figure 8: pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 M methylmercury (solid points) and in an aqueous solution containing 0.265 M methylmercury and 0.265 M valine (open points). Approximately one-half of the actual experimental points are shown.

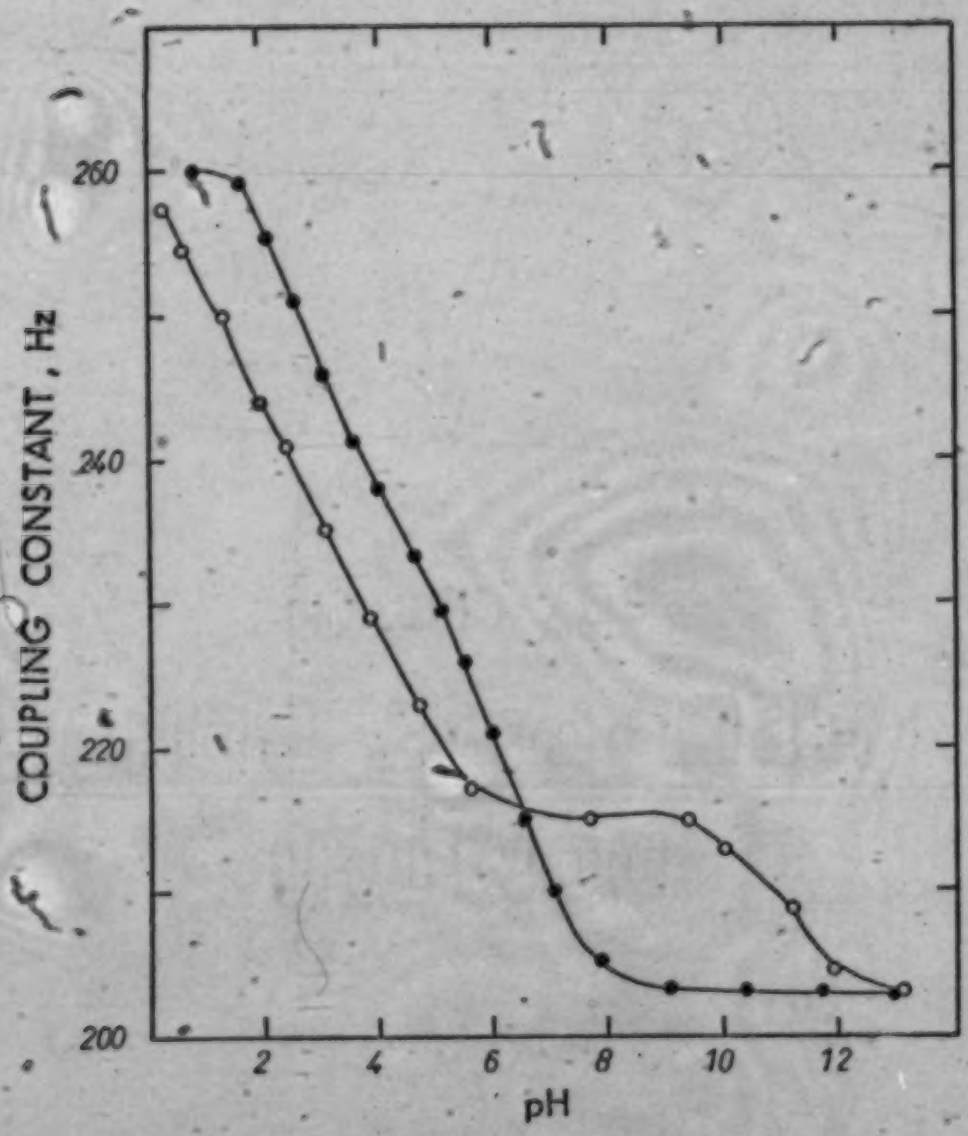


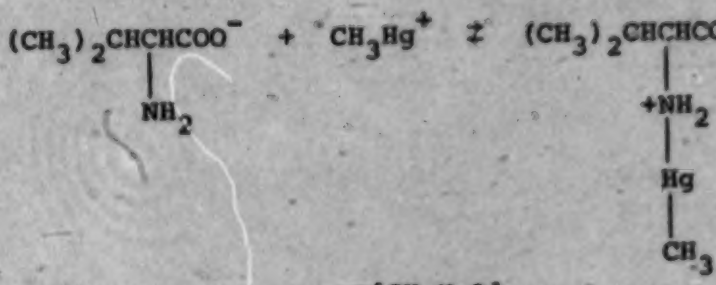
Figure 9: pH dependence of the mercury-proton spin-spin coupling constant of methylmercury in an aqueous solution containing 0.190 M methylmercury (solid points) and in an aqueous solution containing 0.265 M methylmercury and 0.265 M valine (open points). Approximately one-half of the actual experimental points are shown.

$$K_{A2} = \frac{[L^-][H_3O^+]}{[HL^{+-}]} \qquad (29)$$

where $[HL^{+-}]$ represents the zwitterion of valine.

The complexation of methylmercury by the deprotonated carboxyl groups of several carboxylic acids was studied by Libich and Rabenstein (35). These workers found that a carboxyl complex forms over the pH range 1 - 9 with the amount of complex formation being strongly pH dependent due to protonation of the ligand at the lower end of this pH range and formation of methylmercuric hydroxide at the high pH end. It was found in a similar study of the complexation of methylmercury by amines that some methylmercury-amine complex forms over the pH range 2 - 13 and that again the extent of complex formation is pH dependent due to protonation of the ligand and formation of methylmercuric hydroxide (58). The species distribution determined as a function of pH in the previous studies indicates that in molecules containing both carboxyl and amino groups, complexation by the carboxyl group will be predominant at pH less than 3 while at pH greater than 8, binding to the amino group will be most important. The NMR titration curves presented in Figures 8 and 9 above pH 8 are very similar to the NMR titration curves for the binding of methylmercury by amines which indicates that, above this pH, methylmercury is binding to the amino group.

The binding of methylmercury by the amino group is described by Equations (30) and (31). The formation constant for this reaction



(30)

$$K_F^N = \frac{[CH_3HgL]}{[CH_3Hg^+][L^-]} \qquad (31)$$

was determined in the general manner outlined in Chapter II Section G using chemical shift data of the type presented in Figure 8. To ensure that only the amino complex is present, data at pH greater than 9 was used. The fractional concentration of amine-complexed methylmercury is given by Equation (32).

$$P_c = \frac{\delta_{OBS} - \delta_f}{\delta_c - \delta_f} \qquad (32)$$

where P_c is the fractional concentration of complexed methylmercury, δ_c is the chemical shift of the complexed methylmercury, δ_f is the shift of free methylmercury, and δ_{OBS} is the observed chemical shift. In this pH range, the "free" methylmercury is essentially all methylmercuric hydroxide whose chemical shift is equal to 2.35 ppm. The

chemical shift of the complexed methylmercury cannot be determined directly because at no pH is all the methylmercury in the complexed form. Consequently an iterative procedure was used to simultaneously evaluate δ_c and the formation constant. Initially a chemical shift of 2.27 ppm was used, which corresponds to the shift of the nearly level portion of the NMR titration curve between pH 7 and 10. P_c was calculated from Equation (32) using this initial value for δ_c for the data at pH greater than 10. P_f was then calculated for each data point from the Equation $P_f = 1 - P_c$. The concentration of the complex at each pH was then calculated from the relation

$$[CH_3HgL] = P_c M_T \qquad (33).$$

where M_T is the total methylmercury concentration. At pH above 10, essentially all of the "free" methylmercury is in the form of the hydroxide and $[CH_3HgOH] = P_f M_T$. The concentration of methylmercury in the cationic form is calculated using K_1 the formation constant of the hydroxyl complex. (See Section B of Chapter III).

$$[CH_3Hg^+] = \frac{[CH_3HgOH]}{(2.34 \times 10^9)[OH^-]} \qquad (34)$$

Since the total methylmercury and valine concentrations are equal, the fractional concentration of free valine is also $1 - P_c$. The concentration of amino-deprotonated

valine is given by

$$[L^-] = \alpha_2 P_f M_T \qquad (35)$$

where α_2 equals $K_{A2}/K_{A2} + [H^+]$ (62). In this manner a value was obtained for the formation constant K_F^N from each data point. Using the average of the individual formation constants and the δ_c used in their calculation a chemical shift titration curve was calculated. δ_c was then changed slightly and a new set of formation constants obtained. The δ_c and log K_F^N which resulted in the smallest standard deviation between calculated and observed chemical shift titration curves are 2.26 ppm and 7.41. The standard deviation of log K_F^N is 0.01. The formation constants calculated from individual data points using the value 2.26 ppm for the chemical shift, δ_c, are given in Table III.

The formation constant for the complexation of methylmercury by the carboxylic acid group of zwitterionic valine as defined by Equations (36) and (39) was evaluated from chemical shift data

$$(CH_3)_2CHCH(NH_3^+)COO^- + CH_3Hg^+ \rightleftarrows (CH_3)_2CHCH(NH_3^+)COOHgCH_3 \qquad (36)$$

$$K_F^O = \frac{[HLHgCH_3^+]}{[HL^{+-}][CH_3Hg^+]} \qquad (37)$$

Table III. Calculated Values for K_F^N

pH	log K_F^N
10.50	7.41
10.80	7.41
11.00	7.40
11.30	7.43
11.50	7.40
11.80	7.41

at pH less than 3. The calculation of K_F^O is complicated by the fact that, in this pH region, the "free" methylmercury is distributed among several forms and, at a given pH, the species distribution depends on the "free" methylmercury concentration. Consequently, δ_f is the weighted average of the chemical shifts of the various forms of "free" methylmercury. Also, as in the determination of K_F^N, δ_c cannot be measured directly. K_F^O was evaluated by an iterative procedure similar to that used in the evaluation of K_F^N, with the addition of an iterative procedure for the evaluation of δ_f for the specific "free" methylmercury concentration. In the iterative procedure for evaluating δ_f, the concentrations of the various "free" methylmercury species were calculated for the "free" methylmercury concentration predicted initially using the value of δ_f at the given pH for the 0.190 M methylmercury solution (Figure 4) in Equation (32). From these concentrations a new value of δ_f is obtained which is then used to predict a new value for the "free" methylmercury concentration. The iterations were continued until the "free" methylmercury concentration remained constant. In each iteration, the concentration of the cationic form of methylmercury for a particular "free" methylmercury concentration was calculated by

$$\frac{2K_1K_2}{[H^+]}[MeHg^+]^2 + \frac{(1 + K_1)}{[H^+]}[MeHg^+] - (MeHg)_{free} = 0 \qquad (38)$$

which is derived from Equations (20) and (22). The concentrations of $(CH_3Hg)_2OH^+$ and CH_3HgOH were then calculated using Equations (21) and (23) and the concentration of the cationic species obtained from Equation (38). Typically, after 4 iterations, successive P_c values differed by less than 0.1 %. The concentration of carboxyl deprotonated valine used in the evaluation of K_F^O was calculated from the relation

$$[HL^{+-}] = \alpha_1 P_f M_T \qquad (39)$$

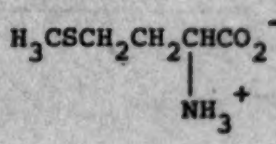
where $\alpha_1 = K_{A1}/K_{A1} + [H^+]$ (62) and M_T is the total methylmercury concentration. The values of δ_c and log K_F^O which resulted in the smallest standard deviation between calculated and observed chemical shift titration curves were 2.16 ppm and 2.7. The standard deviation of log K_F^O is 0.2. The individual results obtained are given in Table IV. The relative lack of precision in the carboxyl formation constant determination, as compared to the amino formation constant determination, arises from the fact that the observed shift of the methylmercury does not differ greatly from the shift of the free methylmercury in the pH region 1-5 whereas in the high pH region, where the amino complex forms, the difference between the observed and free shifts is quite large.

Table IV. Calculated Values of K_F^O

pH	log K_F^O
1.00	3.04
1.50	2.79
2.00	2.65
2.50	2.66
3.00	2.80

2. The Binding of Methylmercury by Methionine

The structural formula of methionine in the zwitterionic form is



II

Natusch and Porter found by PMR that at low pH, Hg(II) was bound by the thioether group (63,64) which suggests that the thioether group might also bind methylmercury. The $-CH_2CH_2CH-$ protons of methionine form an AM_2N_2 spin system and the methine proton gives rise to an approximate triplet. The shift of the central peak of this triplet was used to monitor interactions at the carboxylic acid and amino groups. While this may not represent the exact shift of the methine proton, it is a parameter which is sensitive to changes in the electronic environment due to deprotonation or complexation reactions. The methyl protons give rise to a sharp singlet.

The curves through the solid points in Figure 10 represent the shifts of the methine and methyl protons in a 0.150 M methionine solution, while the curves through the open points represent the shifts of these protons for a solution containing 0.160 M methylmercury. The curves through the solid points in Figure 11 and 12 represent the shift and coupling constant of methylmercury in a solution containing 0.190 M methylmercury while the curves

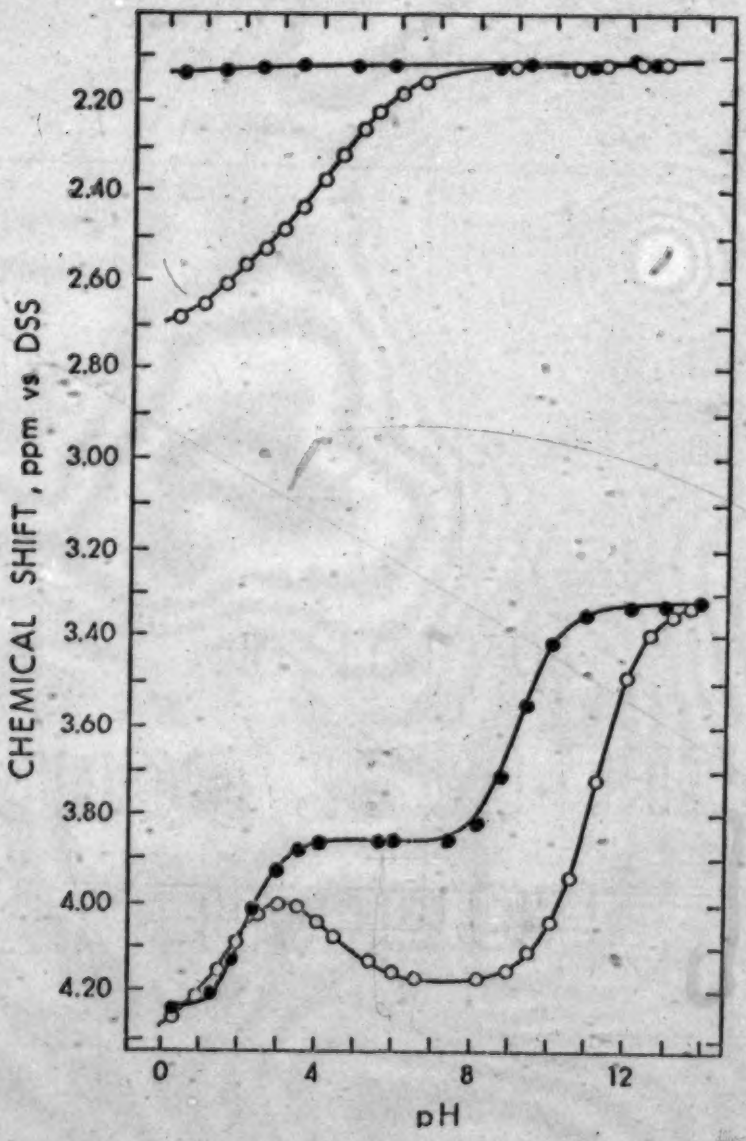


Figure 10: pH dependence of the methyl (upper curves) and methine (lower curves) protons of methionine in aqueous solutions containing 0.150 M methionine (closed points) and 0.160 M methionine and 0.160 M methylmercury (open points).

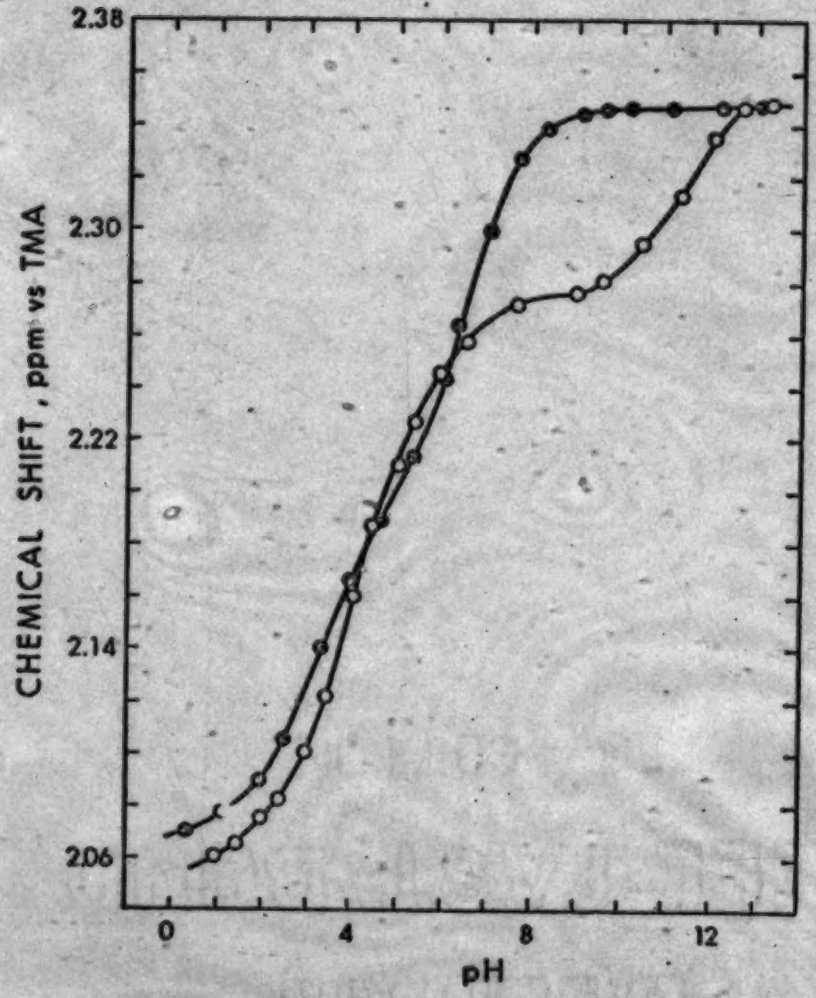


Figure 11: pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 M methylmercury (solid points) and in an aqueous solution containing 0.160 M methylmercury and 0.160 M methionine (open points).

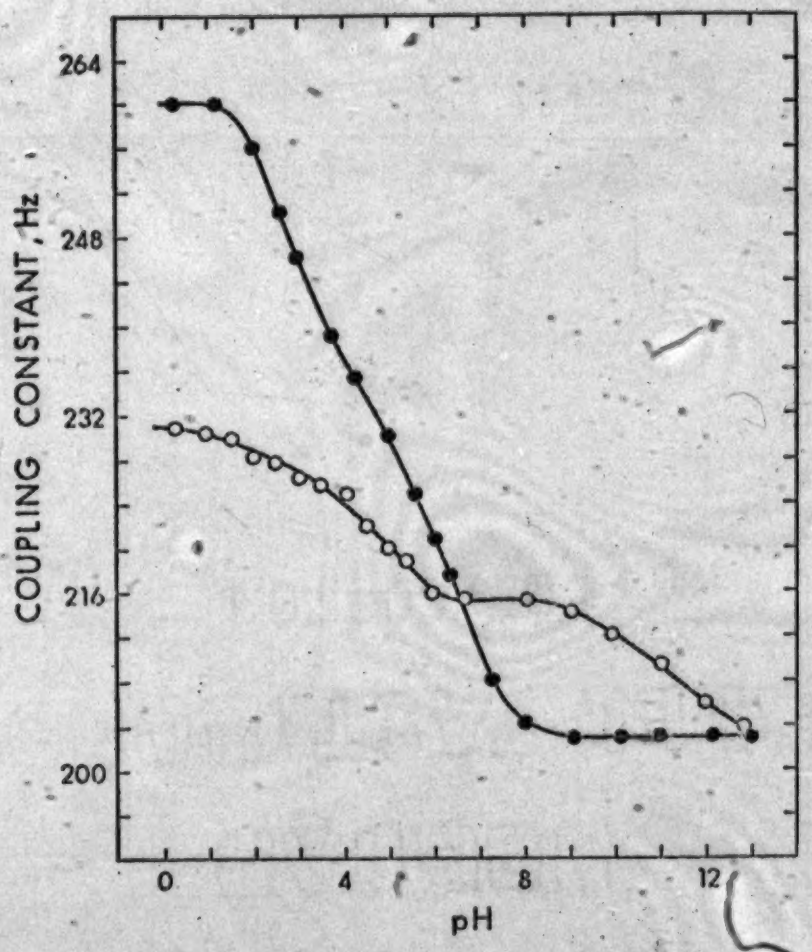


Figure 12: pH dependence of the methylmercury mercury proton spin-spin coupling constant in aqueous solutions containing 0.190 M methylmercury (solid points) and 0.160 M methylmercury and 0.160 M methionine (open points).

through the open points represent the shift and coupling constant data for the solution containing 0.160 M methylmercury and 0.160 M methionine.

It is well known that the shifts of carbon-bonded protons close to acidic functional groups are sensitive to the deprotonation of that functional group (65). Since the methine proton is close to both functional groups, its chemical shift, as shown in Figure 10 for the 0.150 M methionine solution, moves upfield as the carboxyl group is deprotonated in the pH range 0-4, remains relatively constant in the pH range 4-7, and then shifts further upfield in the pH region 7-11 as the amino group is deprotonated. In the pH region 0-4 the observed shift is the weighted average of the shifts of the forms in which the carboxyl is protonated and deprotonated as described by

$$\delta_{OBS} = P_p\delta_p + P_d\delta_d \qquad (40)$$

where the subscripts p and d refer to the protonated and deprotonated forms. Substitution of $P_p + P_d = 1$ into Equation (40) results in the following Equations for P_p and P_d.

$$P_p = \frac{\delta_{OBS} - \delta_d}{\delta_p - \delta_d} \qquad (41)$$

$$P_d = \frac{\delta_{OBS} - \delta_p}{\delta_d - \delta_p} \qquad (42)$$

K_{A1} was then calculated from each of the observed shifts in this pH range using Equation (43). Similarly the

$$K_{A1} = \frac{[H^+]P_d}{P_p} \qquad (43)$$

observed chemical shifts in the pH range 7-11 are the weighted average of the shifts of amino protonated and deprotonated forms from which the acid dissociation constant K_{A2} for the amino group was calculated. The values for pK_{A1} and pK_{A2} are 2.0 and 9.2, both with a standard deviation of 0.1. The shift of the methyl protons in methionine is essentially independent of pH over the pH range 0-14 which indicates that it is not affected by the state of protonation of the distant amino and carboxyl groups and that the thioether group is not protonated even under very acidic conditions.

At pH less than 2 the chemical shift of the methine proton is not affected by the presence of methylmercury (see Figure 10), whereas the methyl protons are shifted downfield which indicates that, in this pH range, methylmercury is bonded to the thioether group. As the pH is increased from pH 2 the chemical shift curves given in Figure 10 for the methine proton in the presence and absence of methylmercury become increasingly different, while the curves for the methyl protons approach each other indicating that the binding site of the methyl-

mercury is shifting away from the thioether group to the other end of the molecule. At pH ~8-9, coordination is exclusively to the amino end. As the pH is increased above 9, the complex begins to dissociate as indicated by the shift of the methine resonance of the methylmercury-containing solution towards that of free methionine. The overlap of the two curves at pH greater than 13.5 indicates complete dissociation of the complex at this pH.

The chemical shift and the ^{1}H-^{199}Hg coupling constant of the methyl protons of methylmercury presented in Figures 11 and 12 also support these conclusions concerning complexation of methylmercury by methionine. Thus the NMR data indicates that a thioether complex forms according to Equation (44) in the low pH region.

$$CH_3SCH_2CH_2\underset{\displaystyle NH_3^+}{\underset{|}{CH}}CO_2H + CH_3Hg^+ \rightleftharpoons CH_3S\underset{\displaystyle +HgCH_3}{\underset{|}{CH_2}}CH_2\underset{\displaystyle NH_3^+}{\underset{|}{CH}}CO_2H \qquad (44)$$

The formation constant for this complex is

$$K_F = \frac{[H_2LHgCH_3^{2+}]}{[H_2L^+][CH_3Hg^+]} \qquad (45)$$

At higher pH, the methylmercury shifts to the other end of the molecule and potentially could be coordinated to either the deprotonated amino or the carboxylic acid groups. By analogy with valine at pH greater than 9, the methylmercury is assumed to be bound by the amino group

as described by reaction (46). The formation constant

$$CH_3SCH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCO_2^- + CH_3Hg^+ \rightleftharpoons CH_3SCH_2CH_2\underset{\displaystyle NH_2\text{-}HgCH_3^+}{\underset{|}{C}}HCO_2^- \qquad (46)$$

for this reaction is given by Equation (47). At intermediate pH values a complex may form in which the methyl-

$$K_F = \frac{[CH_3HgL^+]}{[L][CH_3Hg^+]} \qquad (47)$$

mercury is bonded to the deprotonated carboxyl group, while the amino group is protonated, although it was not possible to identify such a complex in the present work.

The formation constants for the reactions described by Equations (44) and (46) were determined from NMR data. The formation constant for the binding of methylmercury by the thioether group was determined from the chemical shift of the methyl protons of methionine. The chemical shift was measured as a function of the ratio of methionine to methylmercury at pH 0.5 and pH 1.5. For this study each sample was prepared separately with the exact stoichiometry achieved by pipeting methylmercury stock solution and adding a weighed amount of methionine. Only the results at pH 0.5 were ultimately used since preliminary calculations indicated the possibility of significant carboxyl complexation at pH 1.5. The data at pH 0.5 are

presented in Table V. At this low pH, the amino and carboxylate dentates are protonated, as indicated by the shift of the methine proton, and "free" methylmercury exists predominantly as the aquated cation. In all cases a single, averaged set of resonances was observed, indicating exchange between the "free" and complexed forms to be fast on the NMR timescale.

Since the chemical shift of the complexed form of methionine could not be determined directly, the formation constant of the thioether complex was determined from the chemical shift data for the methyl protons of methionine given in Table V by the iterative procedure described previously for the methylmercury valine complexes, with the exception that all of the "free" methylmercury at this pH is present as the cation. Using this procedure, the formation constant was determined to be 87 with a standard deviation of 4 and the chemical shift of the methyl protons of the thioether-complexed methionine to be 2.86 ppm. From the chemical shift data for the methyl protons of methylmercury and the mercury-proton coupling constant data in Table V, the chemical shift of the methyl-protons of methylmercury bonded to the thioether group is calculated to be 1.12 ppm while the mercury-proton coupling constant is found to be 223 Hz.

The formation constant for the amino complex

Table V. PMR Data for Methionine and Methylmercury at pH 6.5 [a].

$[\text{Methylmercury}]_{total}$ M	$[\text{Methionine}]_{total}$ M	δ_{CH_3S} [b]	δ_{CH_3Hg} [b]	J_{H-Hg} [c]
0.198	0.199	2.705	1.110	230
0.199	0.219	2.679	1.110	231
0.197	0.236	2.652	1.120	229
0.197	0.256	2.628	1.120	228
0.198	0.276	2.599	1.120	227
0.195	0.285	2.587	1.120	228
0.208	0.0302	2.818	1.100	
0.202	0.595	2.808	1.100	
0.201	0.0991	2.796	1.100	237
0.202	0.148	2.768	1.000	

(a) 25°C

(b) ppm vs DSS

(c) Hz

defined by Equations (46) and (47), was determined from chemical shift data for the methine proton of methionine and the methyl protons of methylmercury for the solution containing equimolar amounts of methylmercuric hydroxide and methionine. The procedure used was identical to that used for valine. Only data at pH values greater than pH 9 were used to ensure that the only complex formed was the amino complex. From the chemical shift data for the methine proton, log K_F = 7.55 ± 0.02 and the chemical shift of the methine proton in the complexed species is 4.21 ppm. The chemical shift data for the methyl protons of methylmercury yielded a log K_F of 7.40 ± 0.08 and a chemical shift of 0.91 ppm for these protons when methylmercury is bonded to the amino group. The individual results are given in Tables VI and VII.

Table VI. Formation Constant Values for Amino Complex Calculated from the Shift of the Methylmercury Protons

pH	log K_F
9.00	7.49
9.50	7.40
10.00	7.33
10.50	7.31
11.00	7.43
11.50	7.49
12.00	7.32

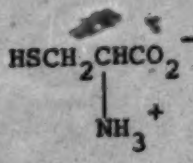
3. The Binding of Methylmercury by Cysteine and Penicillamine.

The binding of methylmercury by the amino acids

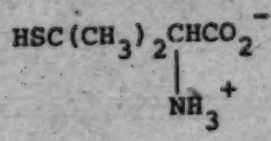
Table VII. Formation Constant Values for Amino Complex Calculated from the Shift of the Methionine Protons.

pH	log K_F
10.50	7.56
10.70	7.53
11.00	7.56
11.35	7.54
11.77	7.55
12.00	7.59
12.86	7.55

cysteine(III) and penicillamine (IV) was studied to



III



IV

characterize the binding of methylmercury by the sulfhydryl group. The chemical shift of the methyl protons of methylmercury in a solution containing 0.150 M methylmercury and 0.150 M cysteine is shown as a function of pH in Fig. 13. Also shown for comparison is the shift of the methyl protons of methylmercury in a 0.190 M methylmercury solution. The mercury-proton spin-spin coupling constants for the same solutions are shown in Figure 14. The chemical shifts of the resonances for the α and β carbon atoms of a 0.15 M cysteine solution and of a solution containing 0.20 M cysteine and 0.20 M methylmercury are shown in Figure 15. The displacement of the chemical shift and coupling constant titration curves of methylmercury when the solution contains cysteine and of the carbon-13 chemical shift titration curves of cysteine when the solution contains methylmercury indicate that methylmercury is complexed by cysteine over the pH range 0-14.

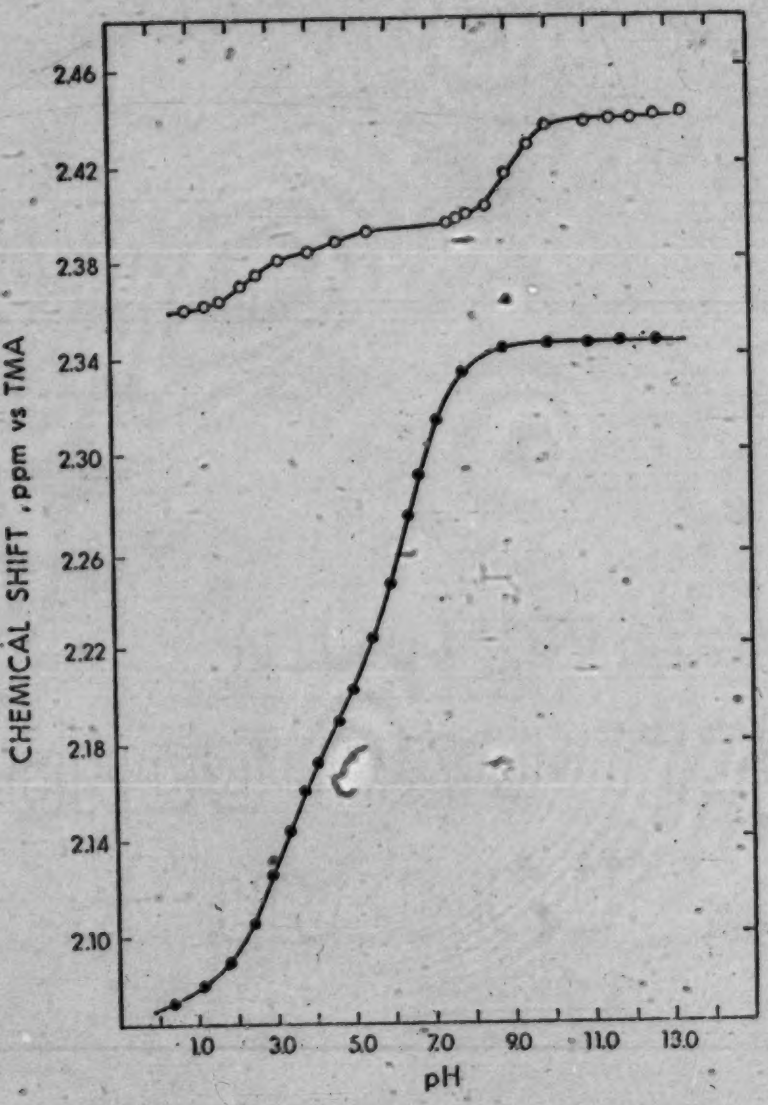


Figure 13: pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 M methylmercury (solid points) and in an aqueous solution containing 0.150 M methylmercury and 0.150 M cysteine (open points).

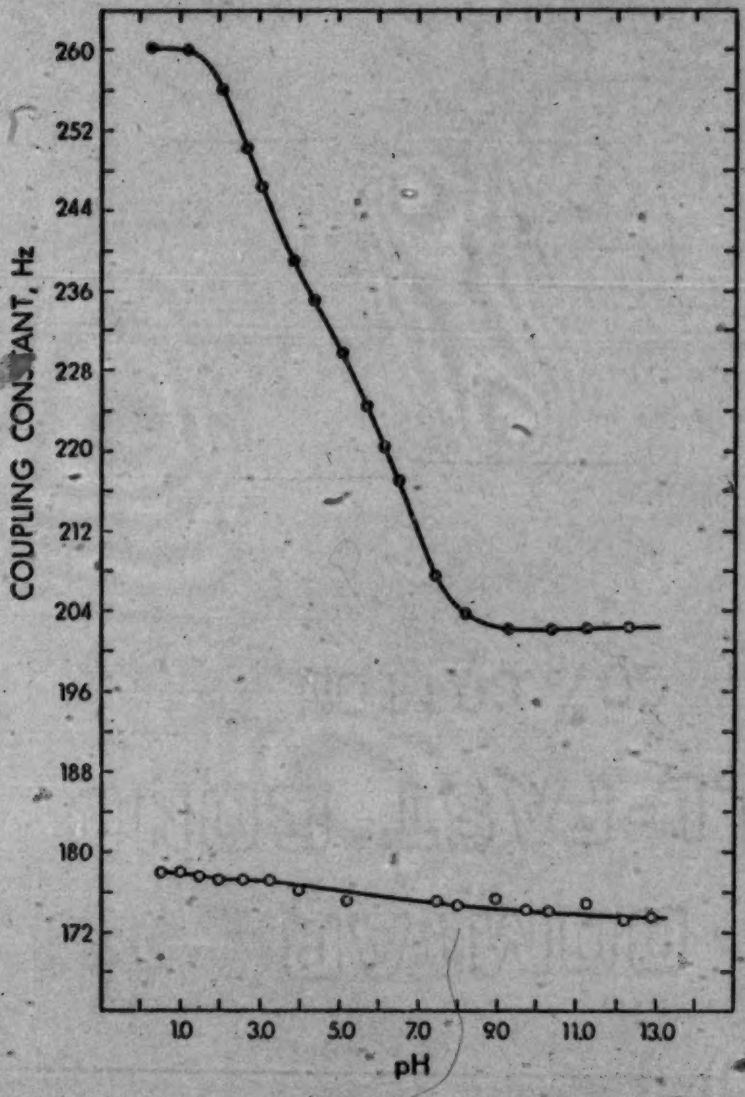


Figure 14: pH dependence of the mercury-proton spin-spin coupling constant of methylmercury in an aqueous solution containing 0.190 M methylmercury (solid points) and in an aqueous solution containing 0.150 M methylmercury and 0.150 M cysteine (open points).

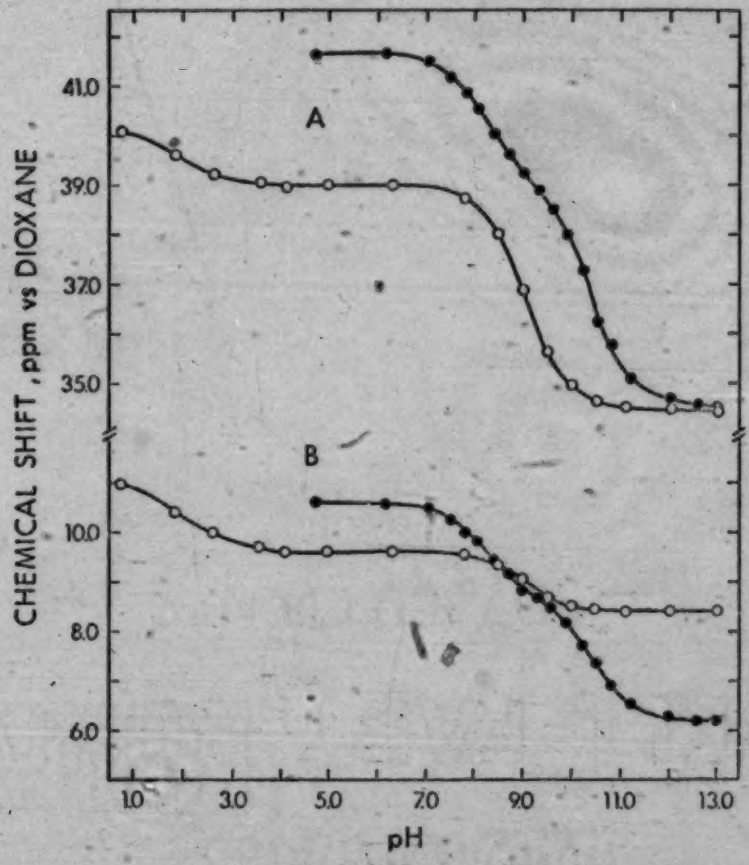


Figure 15: pH dependence of the chemical shifts of the $-CH_2S$ (A) and CHN (B) carbon atoms of cysteine in an aqueous solution containing 0.201 M cysteine (solid points) and in an aqueous solution containing 0.200 M cysteine and 0.200 M methylmercury (open points).

To identify which of the three potential binding sites is involved in methylmercury complexation, the binding of methylmercury by cysteine was studied at pH 13 since the previous studies with valine indicated that the amino and carboxyl groups are not methylmercury-complexed at this pH. In Figure 16, the chemical shift of the methyl protons of methylmercury is given as a function of the cysteine to methylmercury mole ratio. Also shown for comparison are the results of a similar experiment with S-methylcysteine. These results indicate that of the 3 potential binding sites of cysteine, the sulfhydryl group is the binding site involved in the complexation of one-coordinate methylmercury at high pH. Comparison of the low pH region of the titration curves used in the study of the binding of methylmercury by valine (Figures 8 and 9) with the low pH region of Figures 13 and 14 indicates that the sulfhydryl group is the binding site over the pH range 0-13. The mole ratio study also clearly indicates that only one methylmercury is bound by the sulfhydryl group of cysteine at this pH.

In the pH region 5-13, the chemical shifts of the α and β carbon resonances of the 0.150 M cysteine solution change as a result of deprotonation of the amino and sulfhydryl groups (Figure 15); acid dissociation constants for these deprotonation reactions are derived from the carbon-13 chemical shift data in Chapter VIII.

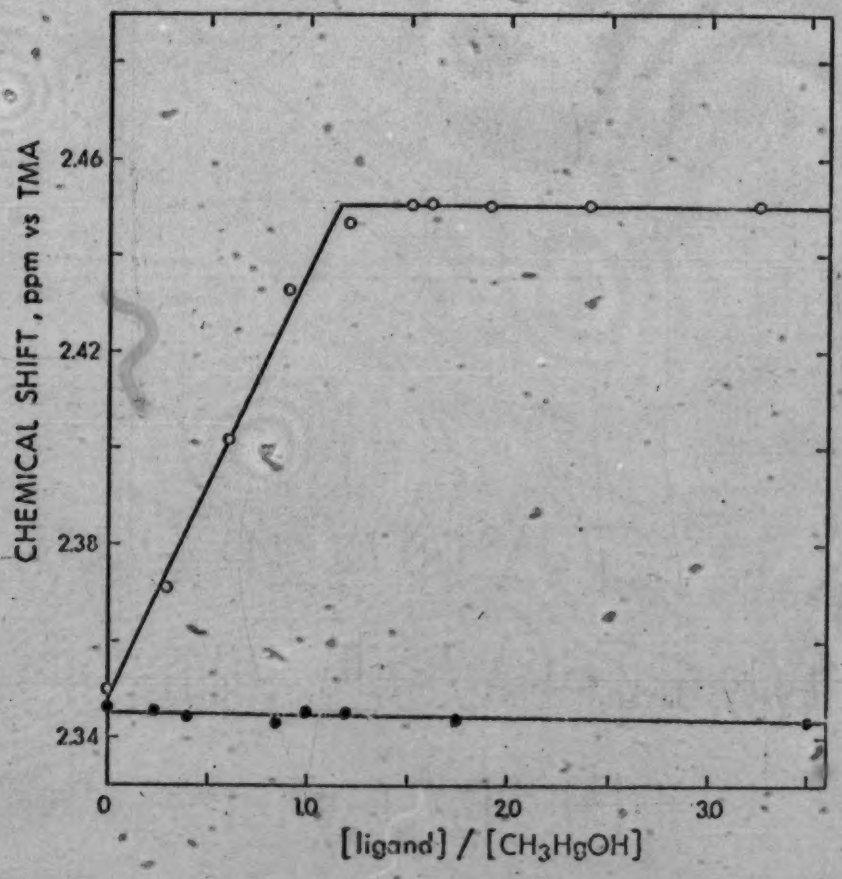


Figure 16: Chemical shift of the methyl protons of methylmercury as a function of the ratio of cysteine to methylmercury (open points) and the ratio of S-methylcysteine to methylmercury (solid points). pH = 13.0.

Assuming the sulfhydryl group to be deprotonated in the cysteine complex of methylmercury, one can attribute the pH dependence of the chemical shifts of the cysteine carbons in the solution containing 0.20 M cysteine and 0.20 M methylmercury (Figure 15) to variations in the state of protonation of the amino and carboxyl groups. Using Equations (41) and (42), the pK_{A1} for the acid dissociation of the carboxyl group of methylmercury-complexed cysteine, defined by Equation (48)

$$CH_3HgSCH_2\underset{\displaystyle NH_3^+}{\underset{|}{C}}HCO_2H + H_2O \rightleftharpoons CH_3HgSCH_2\underset{\displaystyle NH_3^+}{\underset{|}{C}}HCO_2^- + H_3O^+ \qquad (48)$$

was determined to be 1.95 ± 0.05 from the chemical shift data in the pH region 1-5 and the pK_{A2} for the acid dissociation of the amino group defined by Equation (49) was found to be 9.05 ± 0.04 from the chemical shift data in

$$CH_3HgSCH_2\underset{\displaystyle NH_3^+}{\underset{|}{C}}HCO_2^- + H_2O \rightleftharpoons CH_3HgSCH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCO_2^- + H_3O^+ \qquad (49)$$

the pH range 7-11. The chemical shift of the methyl protons of methylmercury in the solution containing 0.20 M methylmercury and 0.20 M cysteine (Figure 13) changes with pH in those pH ranges where the carboxyl and amino groups are being titrated and presumably also reflects the state of protonation. The pK_{A2} value determined from the data

in Figure 13 for the amino group of methylmercury-complexed cysteine is 9.0 ± 0.1. The chemical shift data in the pH range 1-5 did not yield a constant value for the pK_{A1} of the carboxyl group, possibly because some protonation of the methylmercury-complexed sulfhydryl group occurs in this pH range. Protonation of the methylmercury-complexed sulfhydryl group is discussed in more detail in Section 4 of this chapter. The pK_A values for the carboxyl and amino deprotonation reactions of methylmercury-complexed penicillamine from the chemical shift of the methine proton of penicillamine are 2.0 ± 0.1 and 9.0 ± 0.1.

The pH dependence of the chemical shift of the methyl protons and of the mercury proton coupling constant of the methylmercury in an equimolar solution of methylmercuric hydroxide and penicillamine is similar to that shown in Figures 13 and 14 indicating that complexation of the methylmercury to the sulfhydryl group of penicillamine is similar to the complexation with cysteine. These two parameters were also measured in basic solutions containing up to 8 M KOH. Under these extreme conditions, the resonance for the methyl protons of methylmercury was between that of "free" methylmercury and the methylmercury in the penicillamine complex, which may indicate partial dissociation of the complex due to competition from hydroxide ion for $CH_3Hg(II)$ or, possibly, formation of a complex of the type $CH_3Hg(OH)(penicillamine)^{2-}$.

The formation constant for the binding of methylmercury by the sulfhydryl group could not be measured by utilizing the competition between H_3O^+ and $CH_3Hg(II)$ for ligand or between ligand and OH^- for $CH_3Hg(II)$ since nowhere in the pH region 1-13 does the hydrogen ion compete successfully with methylmercury for the ligand or hydroxide ion with the ligand for methylmercury. The determination of the formation constant was attempted by using a competing ligand which forms a moderately strong complex with methylmercury. Possibilities suggested by Schwarzenbach and Schellenberg's results (2) are thiosulfate ions (log K_F = 11) and cyanide ions (log K_F = 14.1). To pH 6 and 13 solutions containing equimolar concentrations of methylmercury and cysteine, thiosulfate was added up to a thiosulfate to methylmercury to cysteine ratio of 4 to 1 to 1. Even with a large excess of thiosulfate present, no significant amount of the cysteine complex dissociated to form the thiosulfate complex, as indicated by the absence of a change in the chemical shift of the methyl protons of methylmercury. In the cysteine complex, the resonance for these protons is at 0.74 ppm whereas in the thiosulfate complex it is at 0.97 ppm.

Similar experiments at a pH of 13 using cyanide ion as the competing ligand were somewhat more successful. When the solution contained a 20- to 100-fold excess of cyanide to methylmercury, the resonance for the methyl

protons of methylmercury moved upfield. However, the shift of the methyl protons of methylmercury in CH_3HgCN differs by less than 2 Hz (0.03 ppm) from the chemical shift in the methylmercury-cysteine complex at pH 13. Consequently these results could only be used to estimate the logarithm of the formation constant for the sulfhydryl complex defined by Equations (50) and (51) to be approximately 15.

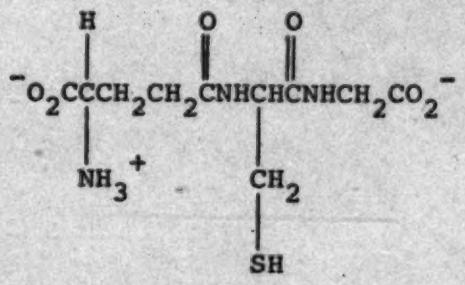
$$^{-}SCH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCO_2^{-} + CH_3Hg^{+} \rightleftarrows CH_3HgSCH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCO_2^{-} \qquad (50)$$

$$K_F^S = \frac{[CH_3HgL]}{[L^-]\,[CH_3Hg^+]} \qquad (51)$$

4. The Binding of Methylmercury by Glutathione

In the previous section it was shown that of the potential coordination sites in the amino acids penicillamine and cysteine, the sulfhydryl group is the strongest coordination site for methylmercury. Presumably the amino and carboxyl groups of sulfhydryl-complexed cysteine and penicillamine are still potential coordination sites for additional methylmercury. Attempts to characterize the binding of methylmercury to these amino and carboxyl groups were unsuccessful because the resonances do not indicate the state of complexation of a single functional group. To further characterize the binding of methylmercury in

such complexes the binding of methylmercury by glutathione-(γ-L-glutamyl-L-cysteinyl-glycine, V) was studied.



V

The methylene protons of the glycyl residue ($NHCH_2CO_2^-$, labelled A in the following discussion), the methylene protons of the L-cysteinyl residue (CH_2-SH, labelled B) and the methine proton of the γ-L-glutamyl residue ($^-O_2C(NH_3^+)CH$-, labelled C) reflect the chemical state of the carboxylic acid group of the glycyl residue, the sulfhydryl group of L-cysteinyl residue, and the α-carboxylic acid and α-amino groups of the L-glutamyl residue. The chemical shifts of protons A, B, and C are shown as a function of pH for a 0.15 M solution of glutathione in Figure 17. The chemical shift of protons A changes in the pH range 1-5 due to deprotonation of the glycyl carboxyl group, that of protons B changes in the pH range 7-11 due to deprotonation of the sulfhydryl group and that of proton C changes in the pH range 1-5 due to deprotonation of the α-carboxylic acid group of the γ-L-glutamyl residue and in the pH range 7-12 due to deprotonation of its α-amino group.

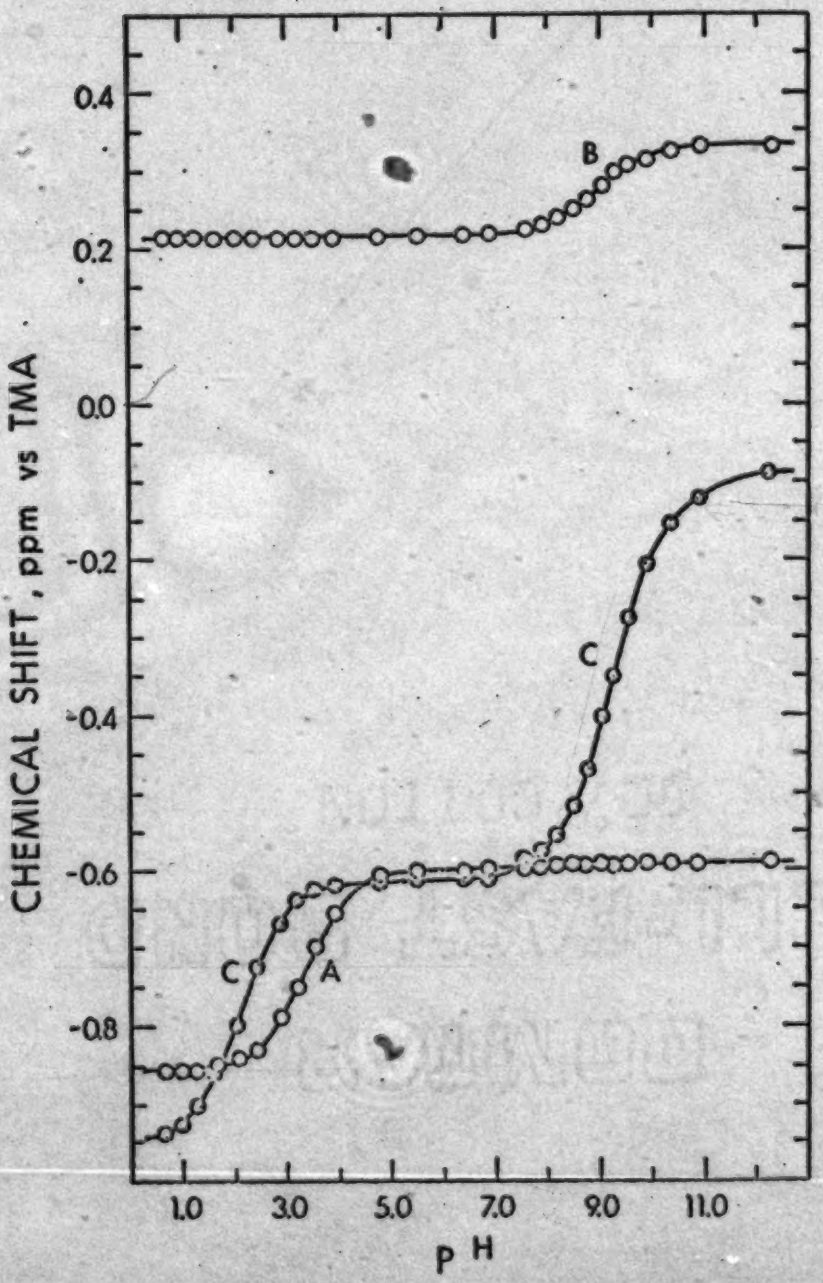


Figure 17: pH dependence of the chemical shifts of the (A) glycyl methylene protons, (B) L-cysteinyl methylene protons, and (C) L-glutamyl methine proton of glutathione. (0.15 M glutathione, T = 25°).

The chemical shifts of protons A, B and C are shown as a function of pH in Figure 18 for a solution containing equimolar amounts of glutathione and methylmercuric hydroxide. A comparison of Figures 17 and 18 shows that the chemical shifts of protons A and C are unaffected by the methylmercury whereas protons B experience a marked pH independent downfield shift. This indicates that methylmercury is bound by the deprotonated sulfhydryl group over the pH range 0-14.

The chemical shift of the methyl protons of methylmercury and the mercury-proton coupling constant for methylmercury in a solution containing equimolar amounts of methylmercury and glutathione are shown as a function of pH in Figures 19 and 20. Also shown for comparison are the chemical shift and mercury-proton coupling constant of a 0.190 M methylmercury solution. The data presented in Figures 19 and 20 also indicate binding of methylmercury by the sulfhydryl group of glutathione.

At pH less than 2, the chemical shift of the methyl protons of methylmercury and the mercury proton coupling constant shift in the direction of the chemical shift and coupling constant of free methylmercury. This could be caused by a slight excess of methylmercury or it could indicate some dissociation of the complex through competition of protons with $CH_3Hg(II)$ for the sulfhydryl group or some protonation of the methylmercury-complexed

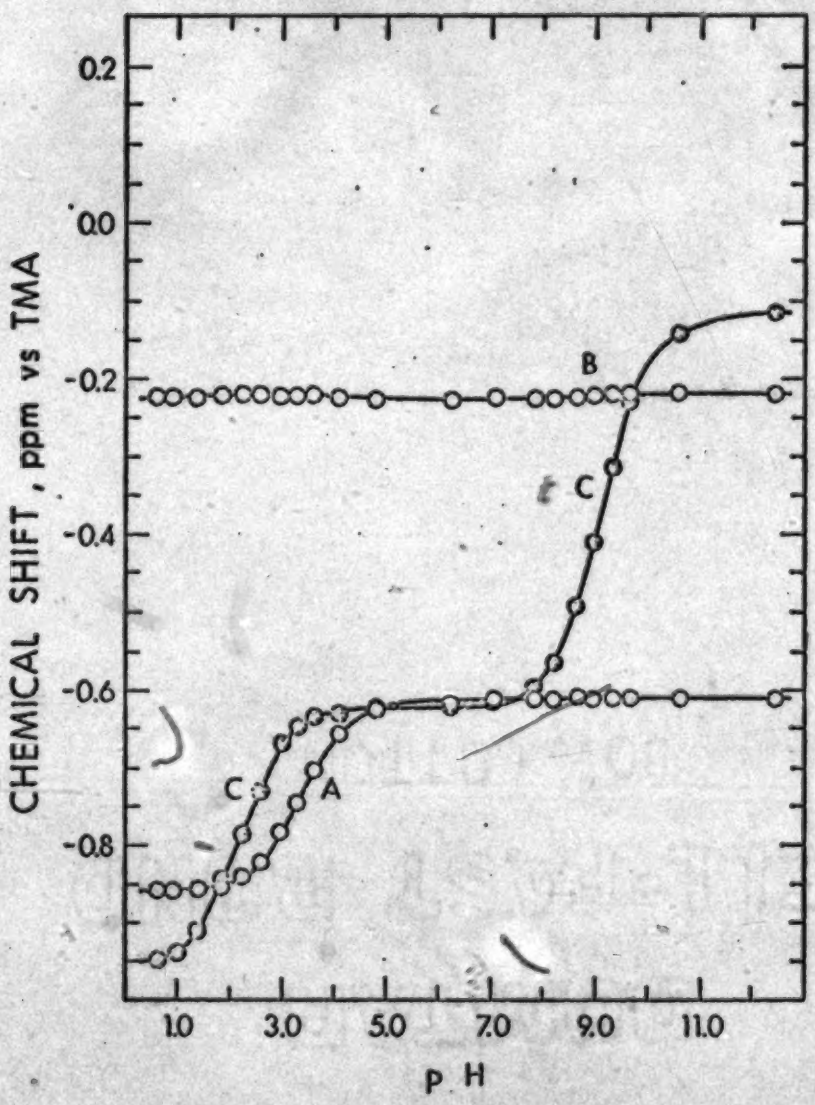


Figure 18: pH dependence of the chemical shifts of the (A) glycyl methylene protons, (B) L-cysteinyl methylene protons, and (C) L-glutamyl methine proton of methylmercury-complexed glutathione (0.15 M CH_3HgOH, 0.15 M glutathione, T = 25°).

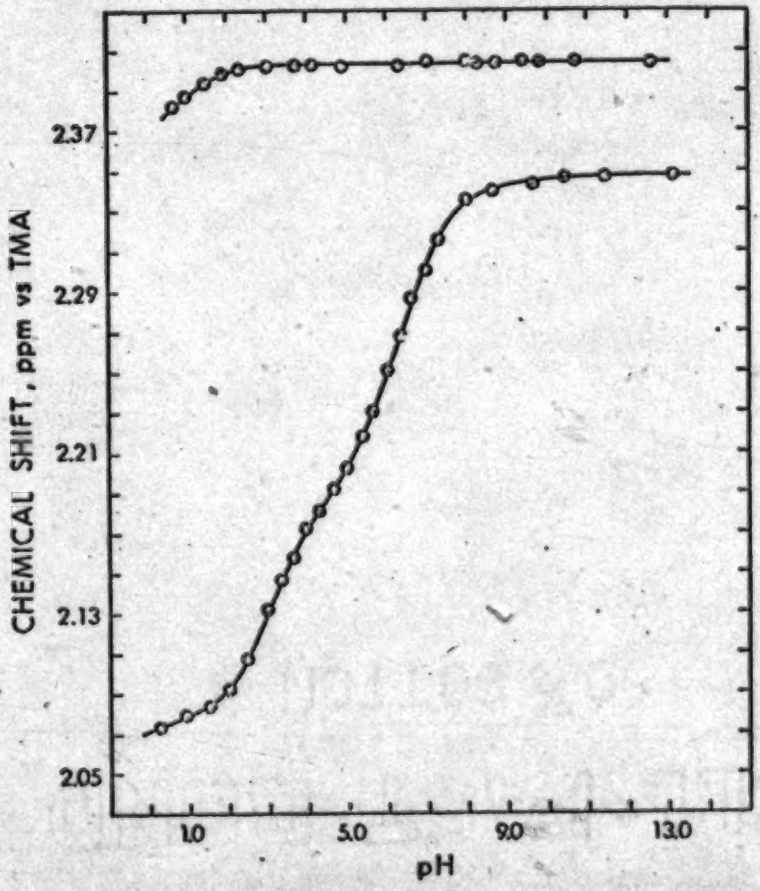


Figure 19: pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 M methylmercury (lower curve) and in an aqueous solution containing 0.150 M methylmercury and 0.150 M glutathione (upper curve).

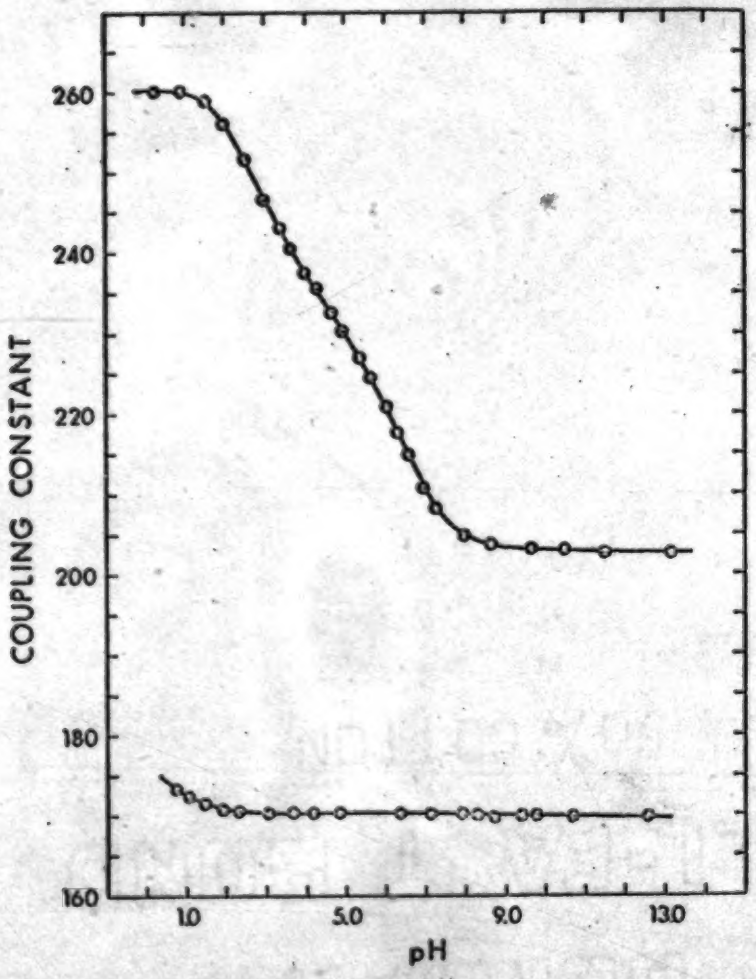


Figure 20: pH dependence of the mercury-proton coupling constant of methylmercury in an aqueous solution containing 0.150 M methylmercury (upper curve) and in an aqueous solution containing 0.150 M methylmercury and 0.150 M glutathione (lower curve).

sulfhydryl. The same results were obtained for an experiment in which the glutathione was present in a 20% excess, indicating that the changes in the chemical shift of the methyl protons of methylmercury and the mercury-proton coupling constant at pH less than 2 in Figures 19 and 20 is not due to an excess of methylmercury. To determine if these changes are due to dissociation of the complex, methionine was added to a pH 0.5 solution containing 0.17 M methylmercury and 0.17 M glutathione. If the methylmercury glutathione complex is partially dissociated, the methionine would react with the free methylmercury to form a thioether complex at this pH which would be indicated by a downfield change in the exchange-averaged chemical shift of the methylmercury protons. No detectable change in the methylmercury chemical shift was observed up to a methionine concentration of 0.34 M. These results suggest that the changes in the chemical shift and coupling constant at pH less than 2 are due to some protonation of the complexed sulfhydryl group. Such a complex would be somewhat similar to the thioether complex formed between methylmercury and methionine, in which the mercury-proton coupling constant is 223 Hz as compared to a coupling constant of 170 Hz in the methylmercury glutathione complex. Thus if protonation of the methylmercury complexed sulfhydryl group is occurring at pH less than 2, the exchange-averaged mercury-proton coupling constant might be expected

to increase as observed.

Since the S-H stretching vibration in glutathione produces a distinctive Raman band in the region of 2580 cm^{-1}, it was thought that evidence for protonation at pH less than 2 might be obtained by Raman spectroscopy. Raman samples were prepared at pH 0.4 of a solution containing 0.42 M methylmercury and 0.42 M glutathione. No S-H stretching vibration was observed, presumably because the concentration of protonated complex is too low to be detected by this method.

To study the binding of additional methylmercury by sulfhydryl-complexed glutathione the chemical shifts of protons A, B and C were measured as a function of pH for a solution containing 0.4 M methylmercury and 0.2 M glutathione. Due to overlap of multiplet patterns, it was not possible to characterize the binding quantitatively with these data. The results suggested, however, that at pH less than approximately 7, two methylmercury ions are coordinated to the sulfhydryl group. To characterize this interaction quantitatively, similar experiments were performed by carbon-13 magnetic resonance spectroscopy.

The carbon-13 magnetic resonance spectrum of a 0.30 M glutathione solution at pH 7.37 is shown in Figure 21. Ten well-resolved resonances are observed, one for each carbon atom of glutathione. The resonances are identified according to the residue and the particular carbon

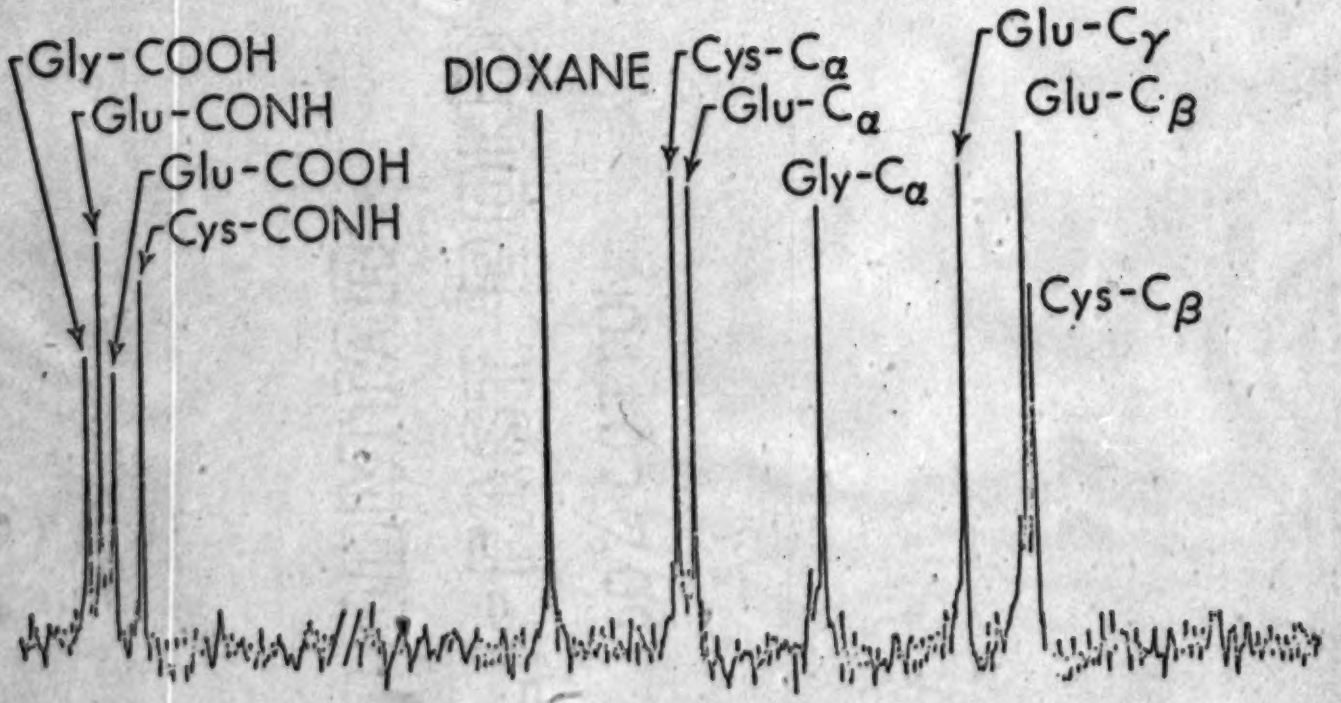


Figure 21: Carbon-13 magnetic resonance spectrum of 0.30 M aqueous solution of glutathione at pH 7.37.

atom within that residue. The chemical shift of each carbon atom is sensitive to deprotonation and complexation reactions occurring at functional groups in close proximity to the particular carbon atom. The chemical shift of the individual carbon atoms is shown as a function of pH in Figure 22. The resonances for the GLY-C_α and GLY-COOH carbons shift as the glycyl carboxylic acid group is protonated. Deprotonation of the sulfhydryl group is reflected in the shifts of the CYS-C_β, CYS-C_α and CYS-CONH carbons. The GLU-C_β, GLU-C_α, GLU-CONH, and GLU-COOH carbon resonances shift as both the carboxyl and amine groups deprotonate.

To elucidate the nature of higher complexes formed from methylmercury-complexed glutathione, the chemical shifts of the carbon atoms of glutathione in solutions containing methylmercury at methylmercury to glutathione ratios of 1:1 and 2:1 were measured as a function of pH. Chemical shift data for the CYS-C_β and GLU-C_β carbon atoms of glutathione are shown in Figure 23. Also shown are the shifts of these carbon atoms in a solution containing only glutathione. Comparison of the chemical shift curve for the CYS-C_β carbon of free glutathione (solid points) with that of the CYS-C_β carbon of a 1:1 methylmercury glutathione solution (half-open points) indicates complexation of the methylmercury by the sulfhydryl group over the pH range studied. The small changes

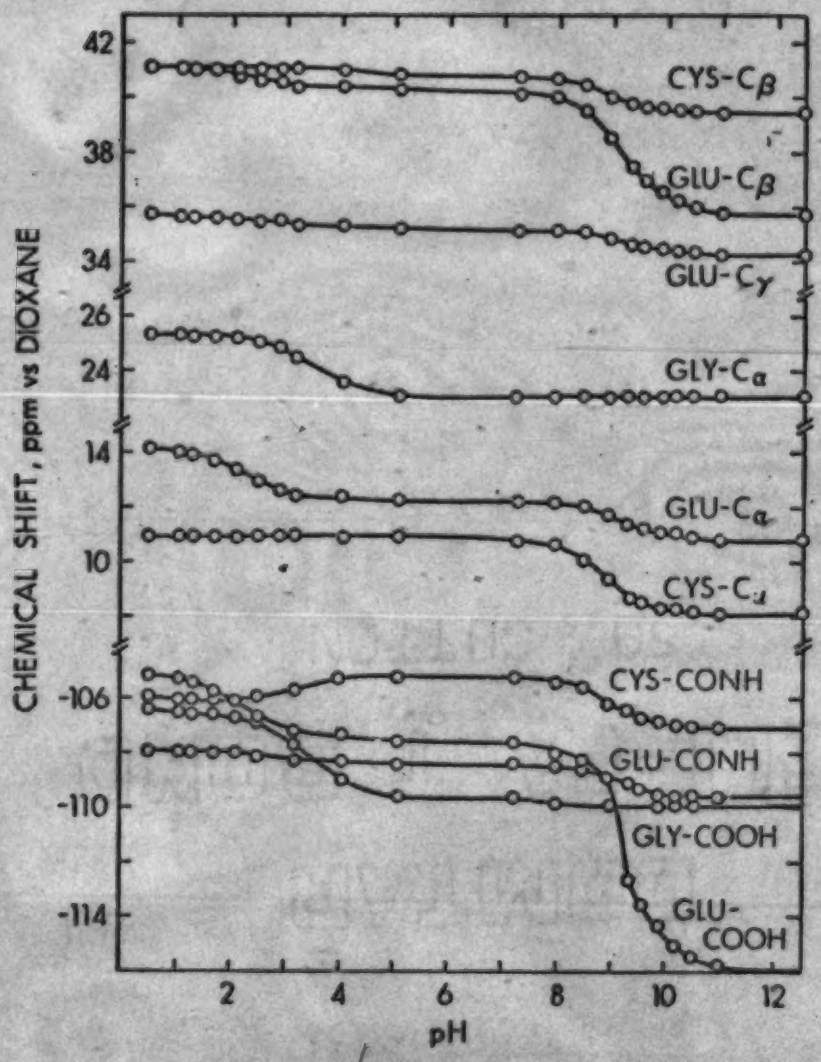


Figure 22: pH dependence of the carbon-13 chemical shifts of the ten glutathione carbon atoms in an aqueous solution containing 0.30 M glutathione.

in the analogous GLU-C_β curves result from changes in the acid dissociation constant of the amino group of methylmercury complexed glutathione when the sulfhydryl group is complexed with methylmercury. Comparison of the CYS-C_β and GLU-C_β chemical shift curves for the 2:1 methylmercury glutathione solution (open points) with those of the 1:1 solution indicates that in the pH range 0-4 two methylmercury cations are bonded to the sulfhydryl group. The mercury-proton coupling constant of the methylmercury in this species is 212 Hz. As the pH is increased above 4, one of the methylmercury cations shifts to the amino group, as indicated by the displacement of the GLU-C_β 2:1 curve from the 1:1 curve. The data in Figure 23 indicate that at pH greater than 9, the second methylmercury is partially dissociated from the glutathione, presumably to form CH_3HgOH by analogy with the pH dependence of the binding of methylmercury by valine.

To characterize these complexes further, mole ratio studies were performed at pH values 1, 4 and 8. The results are presented in Figures 24, 25 and 26. At pH 1, the shift of the GLU-C_β carbon remains constant (Figure 24) indicating no complexation to the amino group, whereas the CYS-C_β shift increases up to a mole ratio of 2 indicating 2 methylmercury cations are coordinated at the sulfhydryl site. The break in the CYS-C_β curve occurs because no averaged resonance could be detected in

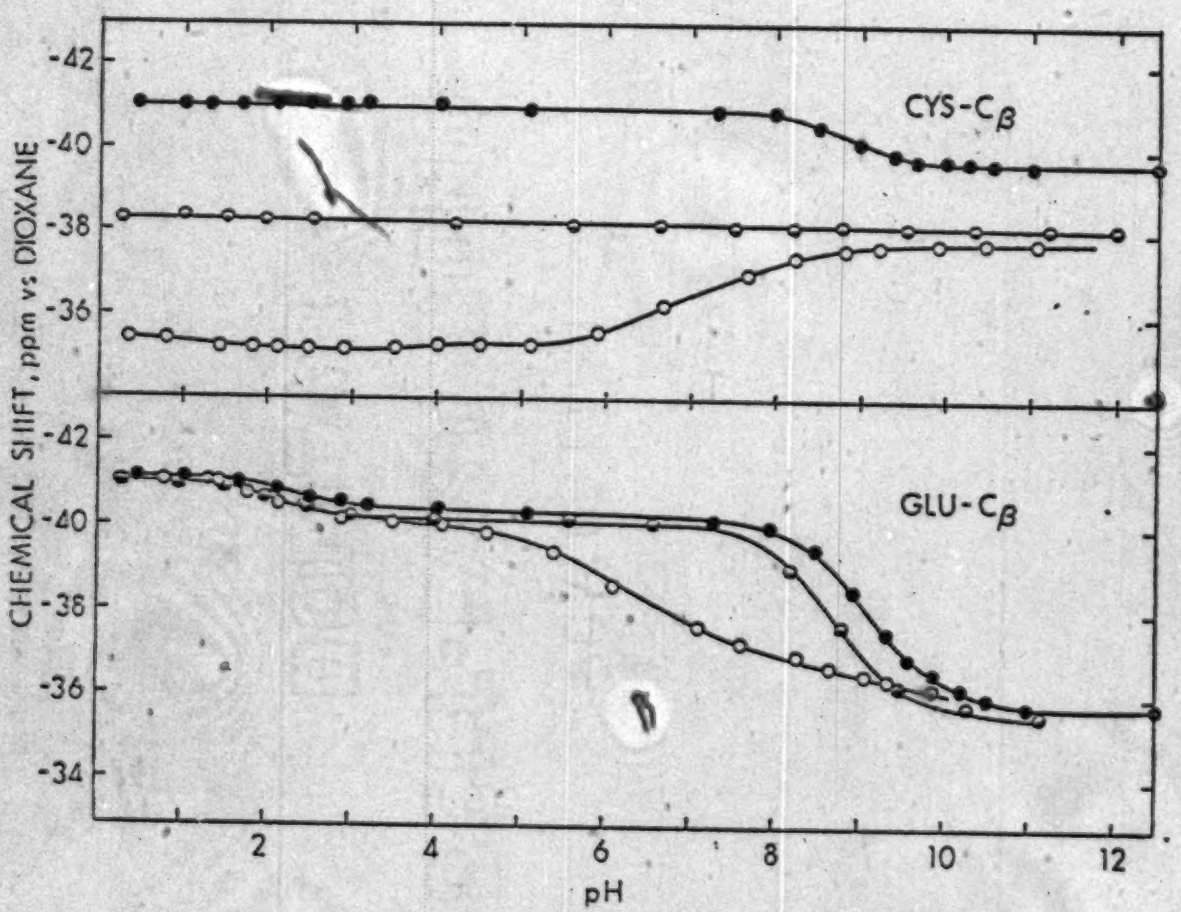


Figure 23: pH dependence of the CYS-C_β and GLU-C_β carbon atoms of glutathione in solutions having methylmercury to glutathione ratios of 0 (- ● -), 1 (- ◒ -) and 2 (- o -). 0.22 M glutathione.

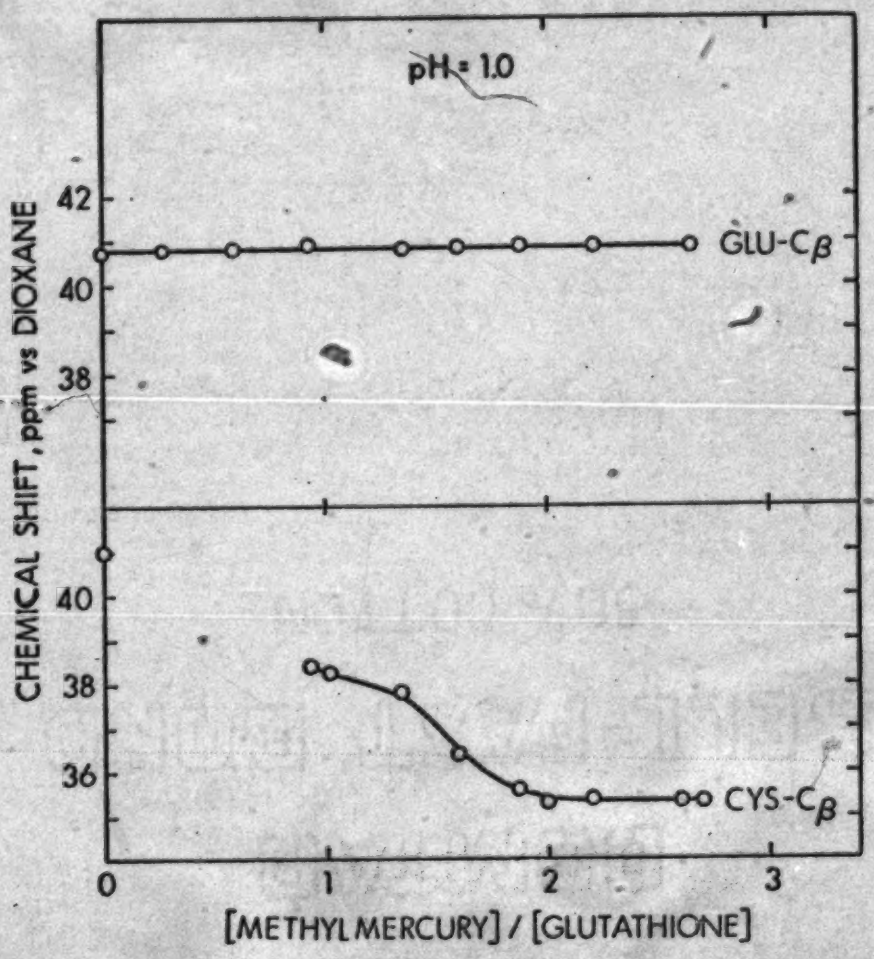


Figure 24: Carbon-13 chemical shifts of the GLU-C_β and CYS-C_β carbon atoms of glutathione as a function of the ratio of methylmercury to glutathione. pH = 1.0.

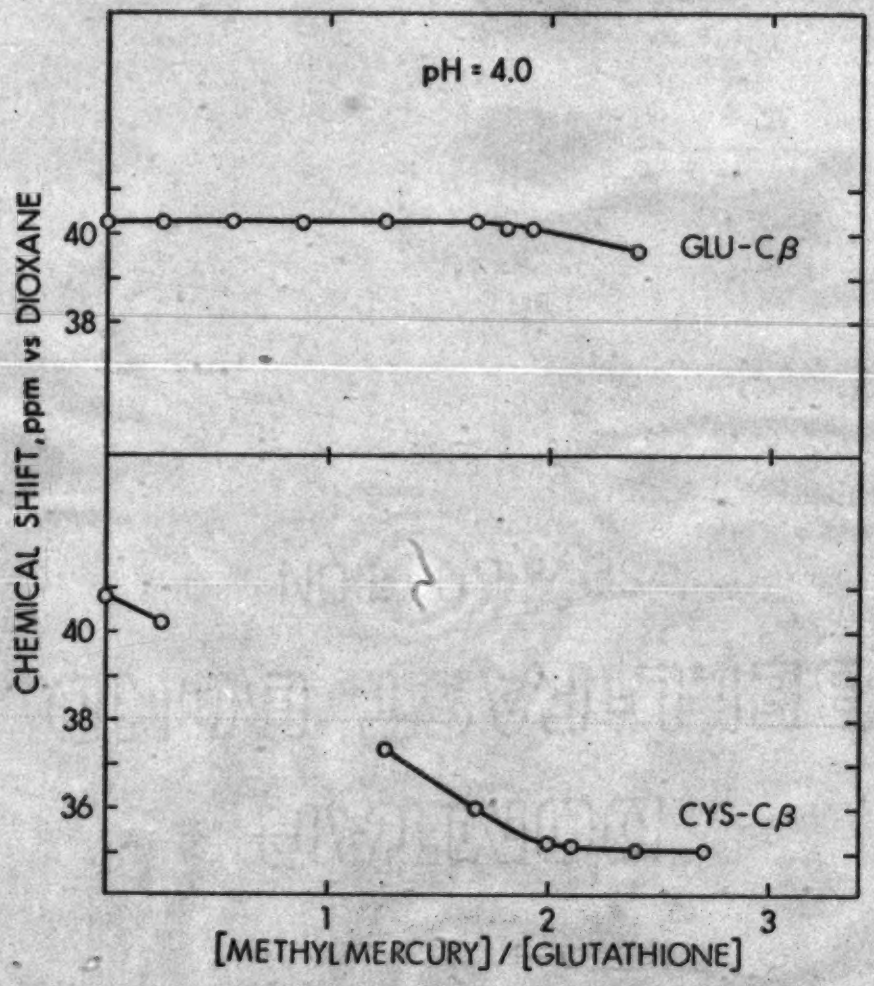


Figure 25: Carbon-13 chemical shifts of the GLU-C_β and CYS-C_β carbon atoms of glutathione as a function of the ratio of methylmercury to glutathione. pH = 4.0.

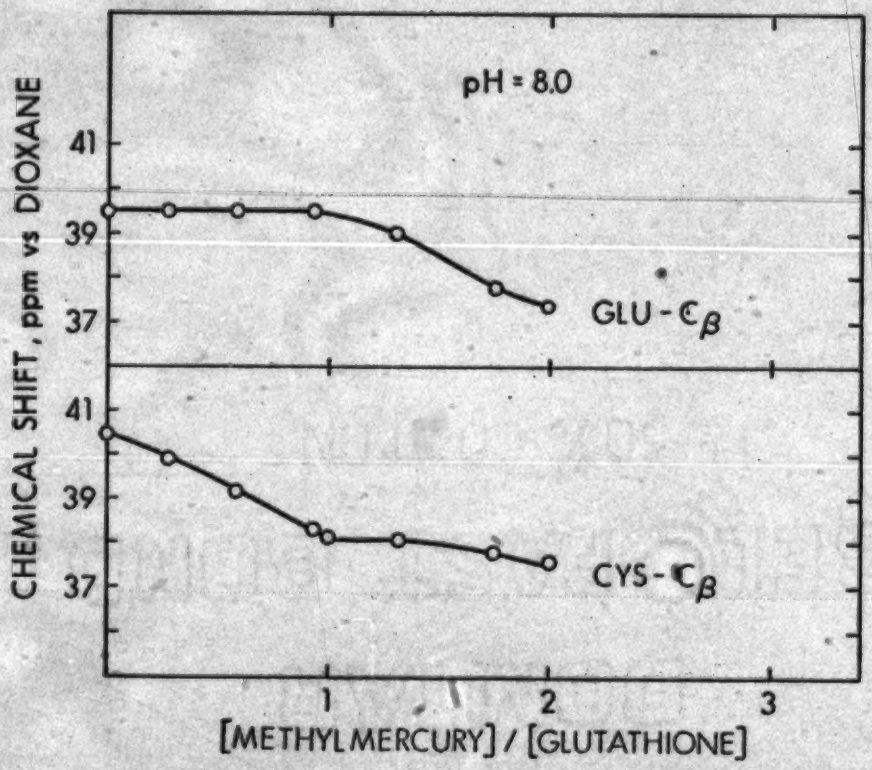


Figure 26: Carbon-13 chemical shifts of the GLU-C_β and CYS-C_β carbon atoms of glutathione as a function of the ratio of methylmercury to glutathione. pH = 8.0.

this region due to exchange broadening. The results in Figure 25 indicate that also at pH 4 the sulfhydryl group is the only coordination site up to a mole ratio of 2. However, the GLU-C_β chemical shift curve indicates that at mole ratios greater than 2 there is some amino coordination. The results given in Figure 26 indicate that, at pH 8, the complexation is somewhat different. Up to a mole ratio of 1, there is only sulfhydryl coordination, but at mole ratios greater than 1 the second methylmercury adds mainly to the amino group as evidenced by the downfield shift of the GLU-C_β carbon.

C. Discussion

1. The Binding of Methylmercury by the Functional Groups of Amino Acids and Peptides.

The NMR results presented in this chapter demonstrate that the carboxylic acid, amino, thioether, and sulfhydryl groups are all potential binding sites for methylmercury with the amount of binding at a particular site being strongly pH-dependent. Of these binding sites, the sulfhydryl complexes methylmercury most strongly as demonstrated by the results in Figure 16 for the mole ratio study with cysteine and S-methylcysteine; in the methylmercury glutathione complex no dissociation was detected over the pH range 0.4 to 13.5. The binding of methylmercury by the carboxyl and amino sites is strongly

pH dependent due to protonation of the coordination site and to formation of the more stable methylmercuric hydroxide. Protonation does not compete with complexation of the methylmercury at the thioether group and the results in Figure 10 indicate that methylmercury binds to the thioether group of methionine at low pH where the "free" methylmercury is present as $CH_3HgOH_2^+$. This indicates that the thioether group has a larger affinity for methylmercury than does H_2O.

The chemical shift data presented in Figures 23, 24 and 25 indicate conclusively that the second methylmercury is complexed by the sulfhydryl group of the 1:1 methylmercury glutathione complex over the pH range 0.4 to 6. The position of the resonance for the methylene protons of the cysteinyl residue of glutathione in a 1:1 methylmercury glutathione solution is essentially independent of pH, indicating that the methylmercury-complexed sulfhydryl group is deprotonated. At pH less than 2, the chemical shift of the methyl protons of methylmercury (Figure 19) and the mercury-proton coupling constant (Figure 20) suggest that there is some protonation of the complex.

Binding of methylmercury by the sulfhydryl-rich protein thionein has been investigated by Chen, Ganther, and Hoekstra (26) who concluded on the basis of spectral changes, that methylmercury has a lower affinity for thionein than does Cd(II). This is opposite to the affinity

order obtained from NMR studies of the binding of these metal ions by the sulfhydryl group of glutathione (27). The greater affinity of thionein for Cd(II) may result from multiple binding of thionein to Cd(II) which does not occur in the Cd(II) complex of glutathione below pH 6 (27) nor in the methylmercury glutathione complex, and presumably not in the methylmercury complex of thionein.

The results in Figure 13 indicate that, upon protonation of the amino group of $CH_3HgSCH_2CHNH_2CO_2^-$ the resonance for the methylene carbon which is two bonds removed from the site of protonation, shifts by 4.58 ppm while that for the methine carbon shifts by only 1.18 ppm even though it is one bond closer to the site of protonation. Similarly, complexation of the ionized sulfhydryl group by $CH_3Hg(II)$ causes the methine carbon, which is separated from the site of complexation by two bonds to shift by 2.22 ppm whereas the resonance for the methylene carbon shifts by only 0.11 ppm even though it is directly bonded to the site of complexation. This is analogous to the relative shifts observed in carbon-13 magnetic resonance spectra upon protonation of simple amino acids (66) and upon complexation of Cd(II) and Zn(II) by the amino group of glutathione (27).

2. The Binding of Methylmercury by Methionine.

The binding of methylmercury by methionine is

of particular interest in view of the previous work on the participation of the thioether group in metal complexes of methionine (63,64,67-69). By a comparison of the formation constants of the glycine and methionine complexes of Zn(II), Li and Manning (67) showed that binding of Zn(II) by methionine is through the amino and carboxylate dentates. In a similar but more extensive study, Lenz and Martell (68) concurred with the conclusions of Li and Manning for Zn(II) and proposed that, of the metal ions Ag(I), Ca(II), Cd(II), Co(II), Cu(II), Hg(II), Mg(II), Mn(II), Ni(II), Pb(II), and Sr(II) only Ag(I) binds to the thioether group. McAuliffe, Quagliano, and Vallarino (69) showed by infrared spectroscopy that, in the solid state at least, binding of several of these and other metal ions by anionic methionine is as proposed by Lenz and Martell (68). More recently, Natusch and Porter (63,64) have demonstrated by proton magnetic resonance spectroscopy that a previously undetected complex in which Hg(II) is bonded solely to the thioether group, forms in acidic solution. Still more recently, Wong, Taylor, Chieh and Carty (70) have reported that in the complex, D,L-methionatomethylmercury, which was isolated from alkaline solution, the methylmercury is bonded to the amino group and there is no interaction between methylmercury cation and the thioether group in the solid state.

The results presented in Figure 10 provide conclusive evidence for methylmercury binding to the thioether group. The formation constant for this complex is relatively small; thus, it dominates the solution chemistry only under very acidic conditions where the amino and carboxylate groups are protonated and are not able to compete with the thioether site for the methylmercury cations. Because of the high degree of specificity in the binding of methylmercury by the thioether group in acidic solution, it may be possible to identify methionine resonances in the PMR spectra of methionine-containing peptides and proteins by observing changes in the spectrum as the peptide or protein is titrated with methylmercury at pH 0.5.

3. pH Dependence of the Binding of Methylmercury.

The extent of complexation of methylmercury by the molecules discussed in this chapter is strongly pH dependent due to protonation of the ligand and reaction of methylmercury with hydroxide ion. Thus the conditional formation constant, defined by Equations (52) and (53)

$$\text{methylmercury}_{\text{free}} + \text{ligand}_{\text{free}} \rightleftarrows \text{methylmercury}_{\text{complex}} \quad (52)$$

$$K_F^C = \frac{[\text{methylmercury}_{\text{complex}}]}{[\text{methylmercury}_{\text{free}}][\text{ligand}_{\text{free}}]} \quad (53)$$

Table VIII. Order of Conditional Stability of Methylmercury Complexes of Ligands in Aqueous Solution.

pH								
1	sulfhydryl	>	thioether	>	H_2O	>	carboxylic acid	> amine
3	sulfhydryl	>	thioether	~	carboxylic acid	>	H_2O	> amine
5	sulfhydryl	>	carboxylic acid	>	thioether	>	amine	~ H_2O, OH^-
7	sulfhydryl	>	amine	>	OH^-	>	carboxylic acid	> thioether
9	sulfhydryl	>	amine	>	OH^-	>	carboxylic acid	> thioether
11	sulfhydryl	>	OH^-	>	amine	>	carboxylic acid	> thioether

is more informative for a given set of solution conditions than formation constants of the type calculated in this chapter since the conditional formation constant indicates directly the extent of complexation for the particular set of solution conditions for which it was derived. The conditional formation constant equals $\alpha\beta K_P$, where α is the fraction of "free" ligand in the form which complexes and β the fraction of "free" methylmercury as the aquated cation for these solution conditions. From the results reported in this chapter, the orders of conditional stability of methylmercury complexes of sulfhydryl, amine, carboxyl, and thioether ligands have been derived as a function of pH and are given in Table VIII. H_2O and OH^- are included to indicate those conditions where the conditional stability constants are so small that little or no complex forms. The information summarized in this Table should be useful when considering which type of complex will predominate in systems containing multifunctional ligands.

4. Relationship between Formation Constant and Mercury-Proton Coupling Constant.

In Chapter I, it was noted that, for a series of carboxylic acids, the absolute magnitude of the mercury-proton coupling constant decreases approximately linearly as both the pK_A of the carboxylic acid and the logarithm

of the formation constant of the methylmercury-carboxylic acid complex increase. Evans et. al. (34) and Sheffold (71) reported a linear decrease in the magnitude of the coupling constant for CH_3HgO_2CR complexes in deuterochloroform with increase in the aqueous solution pK_A of the carboxylic acid. In Figure 27 the absolute magnitude of the mercury-proton coupling constant is plotted as a function of log K_F for thioether, hydroxyl, sulfhydryl and amino complexes of methylmercury. The results plotted in Figure 27 include those obtained in the present work and those obtained in previous studies on these systems. An approximately linear relationship is observed. From a least squares treatment of the data $J = -5.09 \log K_F + 249$. This relationship should be useful in determining the site of complexation in multifunctional ligands.

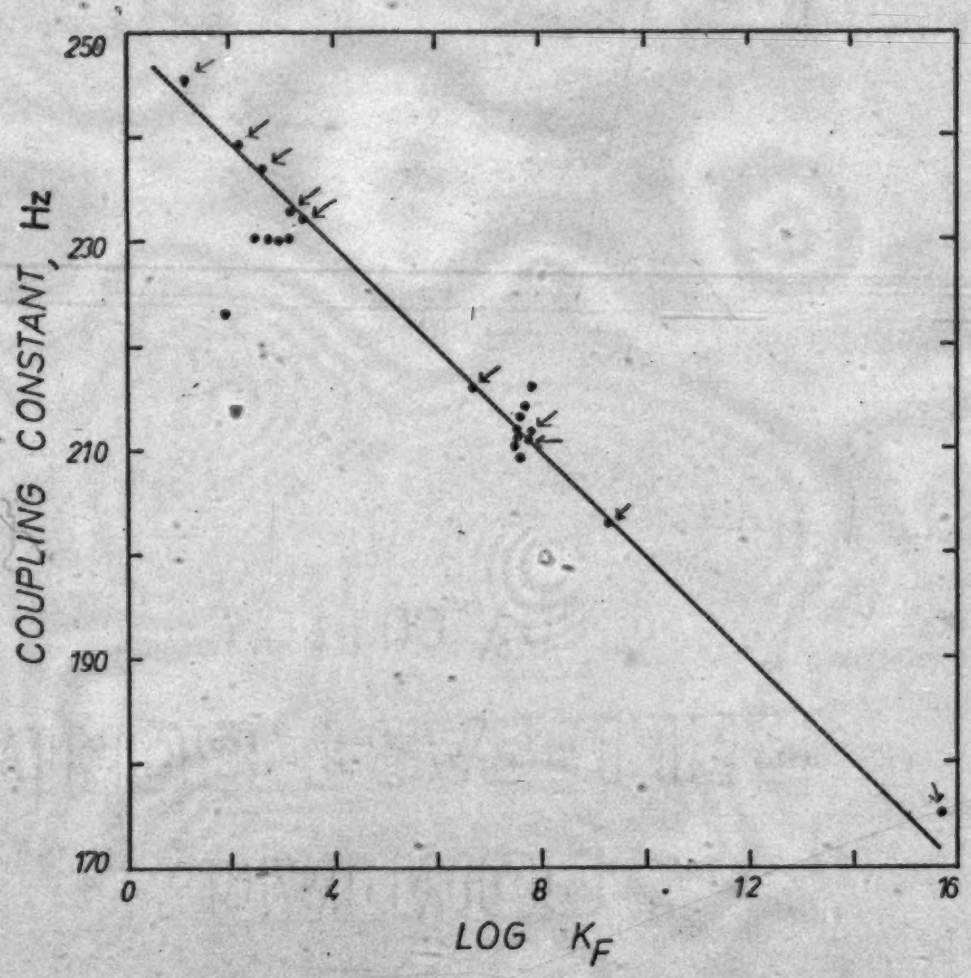


Figure 27: Plot of mercury-proton spin-spin coupling constants versus the logarithm of the formation constants of the methylmercury complex. Values for all ligands studied in this thesis as well as values reported in the literature are included. The least squares analysis was done using the points indicated by arrows.

CHAPTER V

KINETICS OF METHYLMERCURY COMPLEXATION REACTIONS

A. Introduction

In nearly all of the ligand-methylmercury systems presented in Chapter IV, as well as those described in the literature, exchange-averaged spectra were observed for both methylmercury and ligand resonances, indicating exchange between free and complexed forms to be fast on the NMR timescale. This was puzzling, particularly in the case of methylmercury complexes of sulfhydryl-containing ligands. Since the formation constant of a methylmercury complex is equal to the ratio of the formation and dissociation rate constants as defined by Equations (54) and (55) an upper limit for the

$$L^- + CH_3Hg^+ \underset{k_d}{\overset{k_f}{\rightleftarrows}} CH_3HgL \qquad (54)$$

$$K_F = \frac{k_f}{k_d} \qquad (55)$$

dissociation rate constant can be predicted from the formation constant and a diffusion-controlled value for the formation rate constant. Such a calculation for a methylmercury sulfhydryl complex predicts that k_d will be less than approximately 10^{-6} sec^{-1} which corresponds to a mean lifetime of the complex of approximately 10^6 sec.

The change in the chemical shift of the methylene protons of the cysteinyl residue of glutathione upon formation of the methylmercury complex is approximately 0.4 ppm so that separate resonances for the free and complexed forms of glutathione in a solution having a concentration of glutathione greater than methylmercury would be expected if the lifetime of each form were greater than approximately 150 sec. Since exchange-averaged spectra are observed, the reaction represented by Equation (54) must not be the only pathway by which methylmercury and ligand exchange between the free and complexed form. In order to elucidate the exchange kinetics of methylmercury complexes, the exchange kinetics for two systems for which exchange broadening was observed were studied. The results are presented in this chapter.

B. Results

1. The Exchange Kinetics of the Methylmercury Complexes of Methylamine.

Broadening of the methylmercury resonances has been observed in solutions containing amines and some simple amino acids over a limited acidic pH range (58). In the following section of the thesis, results of a study of the exchange kinetics of the methylmercury-methylamine complex are presented.

In a solution containing equimolar concentrations

of methylmercury and methylamine above pH 5, exchange-averaged spectra are observed for both methylmercury and methylamine protons, indicating exchange of both methylmercury and methylamine between the various free and complexed forms is rapid on the NMR timescale. Below pH 5, the central resonances and the two satellite resonances of the methylmercury spectrum are broadened, indicating that exchange of methylmercury between the various forms occurs at a rate which is measurable on the NMR timescale. The amount of broadening is pH dependent. In this pH region, the resonance for the methyl protons of methylamine is also broadened; however, it is difficult to analyze the broadening in terms of exchange of methylamine between free and complexed forms because the methylamine proton exchange kinetics are also slow in acidic solution and cause broadening of the methylamine resonance.

The effect of temperature on the NMR spectrum of methylmercury is shown in Figure 28. The central component of the methyl resonance of methylmercury in a solution containing 0.583 M methylamine and 0.146 M methylmercury at pH 2.65 is shown in Figure 28. At pH 2.65, the predominant "free" methylmercury species are $CH_3HgOH_2^+$ and $(CH_3Hg)_2OH^+$. At 1°C, two methylmercury resonances are observed: the upfield resonance at 0.89 ppm versus DSS, arises from the methylamine-complexed methylmercury, while the downfield resonance at 1.03 ppm is an

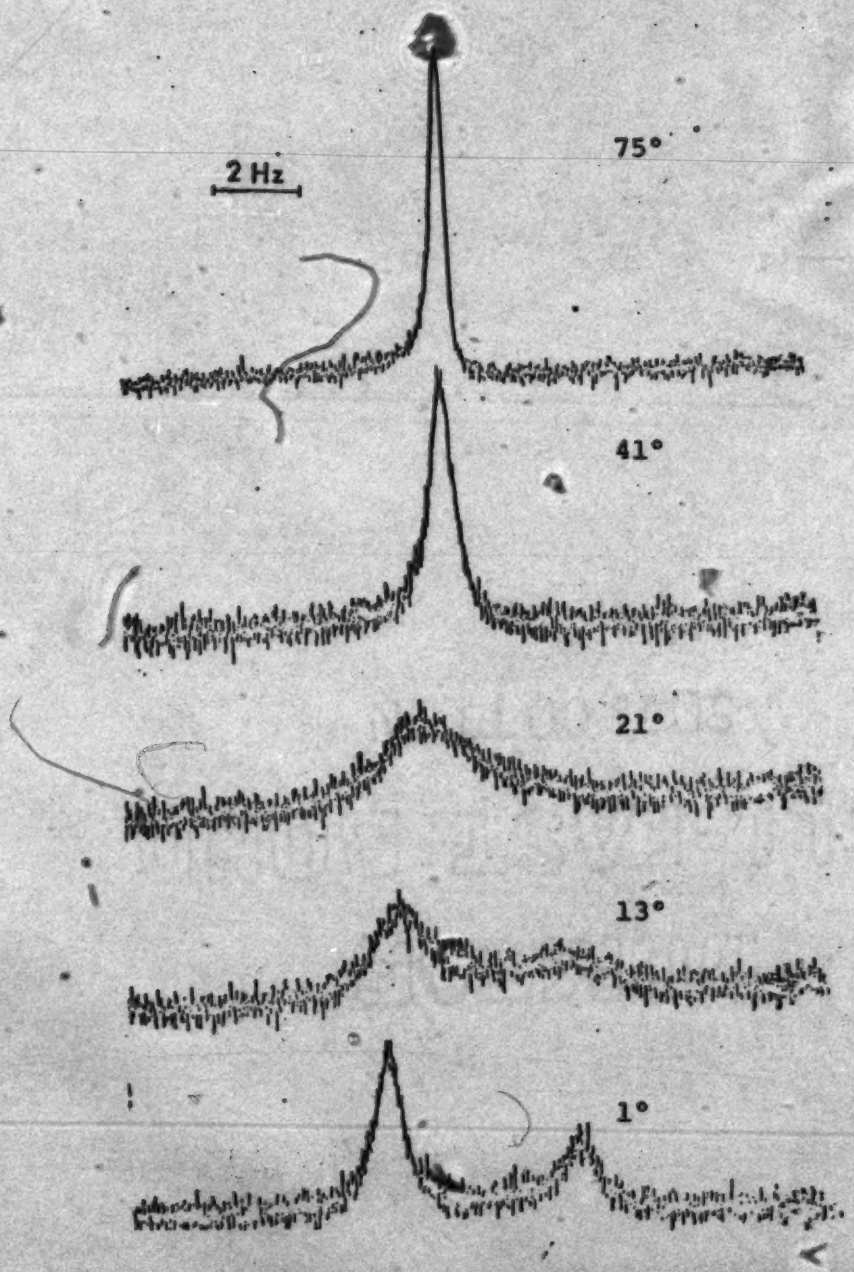


Figure 28: Central resonance of the methylmercury PMR spectrum of a solution containing 0.583 M methylamine and 0.146 M methylmercury as a function of temperature. $\overline{pH}$ = 2.65.

averaged resonance from the "free" methylmercury. Calculation of the fractional concentrations of methylmercury in the free and complexed forms using the reported formation constant (58) for the methylmercury methylamine complex at 25°C indicates that, at this pH, approximately one third of the methylmercury is complexed, which is consistent with the relative intensities of the two resonances at 1°C. Thus at 1°C, the exchange of methylmercury between free and complexed forms is slow on the NMR timescale, but exchange of methylmercury between various "free" forms is fast as indicated by the exchange-averaged "free" methylmercury resonance. As the temperature is increased, the two resonances broaden and coalesce because the rate of exchange of methylmercury between free and complexed forms increases. At 75°C, a single averaged narrow resonance is observed, representing the fast exchange limit.

In the evaluation of the methylmercury exchange kinetics only the exchange broadening of the central methylmercury resonance was used. Broadening of the satellite peaks was also observed but since each satellite constitutes only 8% of the total integrated intensity of the methylmercury resonance, the signal to noise ratio was too low to permit the determination of precise lifetimes from the widths of these resonances.

In the methylmercury-methylamine system there are several exchange reactions occurring simultaneously.

Methylmercury and methyl amine are exchanging between the "free" and complexed forms, possibly by the following pathways.

$$CH_3HgNH_2CH_3^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CH_3Hg^+ + NH_2CH_3 \qquad (56)$$

$$CH_3HgNH_2CH_3^+ + OH^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CH_3HgOH + NH_2CH_3 \qquad (57)$$

$$CH_3HgNH_2CH_3^+ + H^+ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} CH_3Hg^+ + NH_3CH_3^+ \qquad (58)$$

$$CH_3HgNH_2CH_3^+ + \overset{*}{N}H_2CH_3 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} CH_3Hg\overset{*}{N}H_2CH_3 + NH_2CH_3 \qquad (59)$$

In addition, uncomplexed methylmercury is exchanging between its various "free" forms and methylamine is exchanging between its protonated and deprotonated forms by the reaction

$$CH_3NH_3^+ + OH^- \rightleftharpoons CH_3NH_2 + H_2O \qquad (60)$$

Of these reactions, only the reactions represented by Equations (56) - (58) will cause exchange broadening of the methylmercury resonance. Exchange of methylmercury between the various "free" forms is fast, as indicated by exchange-averaged spectra for methylmercury solutions,

while the reaction represented by Equation 59 results in exchange of methylmercury between identical environments and its rate does not influence the width of the resonance line.

The lifetime of the complexed species, τ_c, was calculated from the width of the exchange-averaged central resonance for methylmercury over a range of pH values and concentrations of methylmercury and methylamine at 25°C. The lifetimes were evaluated from the width at half-height using Equation (19) presented in Chapter II. To evaluate the lifetime from the linewidth for a given solution, the populations of the free and complexed forms are required, as are the chemical shifts of the central resonance for methylmercury in these forms. The chemical shift of the complexed form, 2.28 ppm, is known from previous work (60). The populations and the chemical shift of the free form were calculated as described in Appendix I. The results obtained are listed in Table IX. The value used for the linewidth of the "free" methylmercury, 0.38 ± 0.04 Hz, was the linewidth of the methyl resonance of a solution containing only methylmercury. The value used for the linewidth of the complexed species, 0.38 ± 0.04 Hz was that obtained from a pH 8.5 solution of methylamine and methylmercury. At this pH, nearly all of the methylmercury is in the form of the complex.

The inverse of the mean lifetime of the complex

Table IX. Kinetic Data for Methylmercury-Methylamine

pH	[methyl-mercury]	[methyl-amine]	$W^{OBS}_{1/2}$	$\frac{1}{\tau_c}$	P_c	P_f
	M	M	Hz	sec^{-1}		
4.51	0.218	0.218	1.20	88±19	0.282	0.718
4.20	0.218	0.218	1.33	84±15	0.221	0.779
3.73	0.218	0.218	1.50	72±8	0.151	0.849
3.38	0.218	0.218	1.22	83±16	0.096	0.904
4.68	0.218	0.437	1.00	80±26	0.543	0.457
4.23	0.218	0.437	1.96	60±5	0.404	0.596
4.01	0.218	0.437	2.02	60±5	0.339	0.661
3.61	0.218	0.437	2.30	58±4	0.233	0.767
3.29	0.218	0.437	1.68	82±9	0.163	0.834
2.80	0.218	0.437	1.12	116±26	0.085	0.915
4.10	0.175	0.525	1.37	89±11	0.456	0.544
3.77	0.175	0.525	2.27	61±4	0.345	0.655
3.54	0.175	0.525	2.50	60±4	0.275	0.725
3.23	0.175	0.525	2.06	76±6	0.193	0.807
2.92	0.175	0.525	1.43	111±15	0.128	0.872
4.21	0.146	0.583	1.21	82±9	0.560	0.440
3.90	0.146	0.583	1.94	67±6	0.444	0.556
3.68	0.146	0.583	2.65	55±4	0.365	0.635
3.43	0.146	0.583	2.90	56±4	0.282	0.718
3.22	0.146	0.583	2.20	79±6	0.221	0.779
2.97	0.146	0.583	1.60	112±15	0.159	0.841

is related to the rate of decrease in the concentration of the complex by

$$\frac{1}{\tau_c} = -\frac{d[c]}{dt}\frac{1}{[c]} \qquad (61)$$

where [c] is the concentration of the complex. The rate of decrease in the concentration of the complex by the reactions which would cause broadening of the methylmercury resonance [Equations (56) - (58)]

$$\frac{-d[c]}{dt} = k_1[c] + k_2[OH^-][c] + k_3[H^+][c] \qquad (62)$$

Division by [c] leads to the following equation which relates the experimentally measured mean lifetime to the individual rate constants.

$$\frac{1}{\tau_c} = k_1 + k_2[OH^-] + k_3[H^+] \qquad (63)$$

The reaction represented by Equation (58) is unlikely to contribute to the exchange since methylmercury-complexed amine does not possess a lone pair of electrons to promote an electrophilic attack by the proton. The reaction represented by Equation (57) can be shown not to contribute significantly to the methylmercury exchange at the pH values in Table IX by predicting its maximum contribution to $1/\tau_c$ at these pH values. The maximum value for k_2 would be the upper limit for a diffusion-controlled bimolecular rate constant at 10^{10} (72), so

that the maximum contribution of the term $k_2[OH^-]$ at pH 4 is 1. If the rate constant is less than the diffusion controlled value, the contribution would be smaller. The magnitude of this term will decrease as pH decreases. The uncertainties reported for $1/\tau_c$ in Table IX were calculated using only the uncertainty in the observed linewidths. Additional sources of error are the values used for P_c and P_f, the fractional populations of free and complexed forms but it is difficult to estimate the magnitude of this error. This error will lead to the largest uncertainty in $1/\tau_c$ when P_c and P_f are very different from each other. Since $1/\tau_c$ does not show, within the estimated uncertainties, a systematic increase as the pH increases, the reaction represented by Equation (57) must not contribute significantly to methylmercury exchange at the pH's listed in Table IX. Thus Equation (62) reduces to

$$\frac{1}{\tau_c} = k_1 \qquad (64)$$

Since the uncertainty in $1/\tau_c$ due to errors in P_c and P_f is greatest when P_c and P_f are very different from each other, an average value of 70 ± 18 for k_1 was calculated using only that data where P_c is greater than 0.2. From the relationship $K_F = k_{-1}/k_1$, k_{-1} is calculated to be 3×10^9 sec^{-1}. At pH less than 2, the methylmercury resonance is not exchange-broadened since at this pH and

lower little or no complexed methylmercury exists. At pH greater than 5 the rate of exchange of methylmercury becomes fast; presumably because of the contribution of a reaction such as that represented by Equation (57) whose rate is pH dependent.

2. The Exchange Kinetics of the Methylmercury Complexes of Glutathione.

With each of the sulfur-containing ligands studied, PMR spectra were recorded for the methylmercury in solutions having methylmercury to ligand ratios greater than one over the pH range 0-13. In every case, a single exchange-averaged resonance pattern was observed, indicating exchange of methylmercury between the various methylmercury species to be fast on the NMR timescale.

However, the rate of exchange of ligand between "free" and complexed forms in solutions containing an excess of ligand is pH dependent. For the methylmercury-penicillamine system, the line width of the exchange-averaged resonances of a solution having a penicillamine to methylmercury ratio of 2:1 indicated fast exchange except over the pH region 1.7 to 3.5 where a small amount of broadening of the averaged penicillamine methine proton resonance was observed. For the methylmercury-glutathione system, the rate of exchange of glutathione between free and complexed forms in a solution containing an excess of

glutathione is fast at pH greater than 6 as indicated by sharp, averaged resonances in both the proton and carbon-13 spectra. At pH less than 6, the rate decreases and is dependent on pH and glutathione concentration. Due to the complexity of the PMR spectrum of glutathione, the exchange was studied by carbon-13 NMR. The CYS-C_α region of the carbon-13 spectra for a solution containing 0.33 M glutathione and 0.165 M methylmercury is shown as a function of pH in Fig. 29; the CYS-C_β resonance is shifted more upon complex formation and thus is more sensitive to kinetic effects, however overlap of the exchange-averaged CYS-C_β resonance with the GLU-C_β resonance precludes analysis of its lineshape. At these concentrations, the exchange rate is sufficiently slow at pH 2.12 that separate exchange-broadened resonances are observed.

The inverse of the mean lifetime of the methylmercury-glutathione complex is given in Table X for a range of experimental conditions. The mean lifetimes were obtained by matching experimental and computer-simulated spectra. Spectra were simulated as a function of the lifetime of glutathione in each of the environments using the Bloch phenomenological equations as discussed in Chapter II. The uncertainty of the lifetimes, due mainly to the low signal-to-noise ratio for the exchange-broadened spectra, of which those in Figure 29 are typical, is estimated to be $\pm$ 15%.

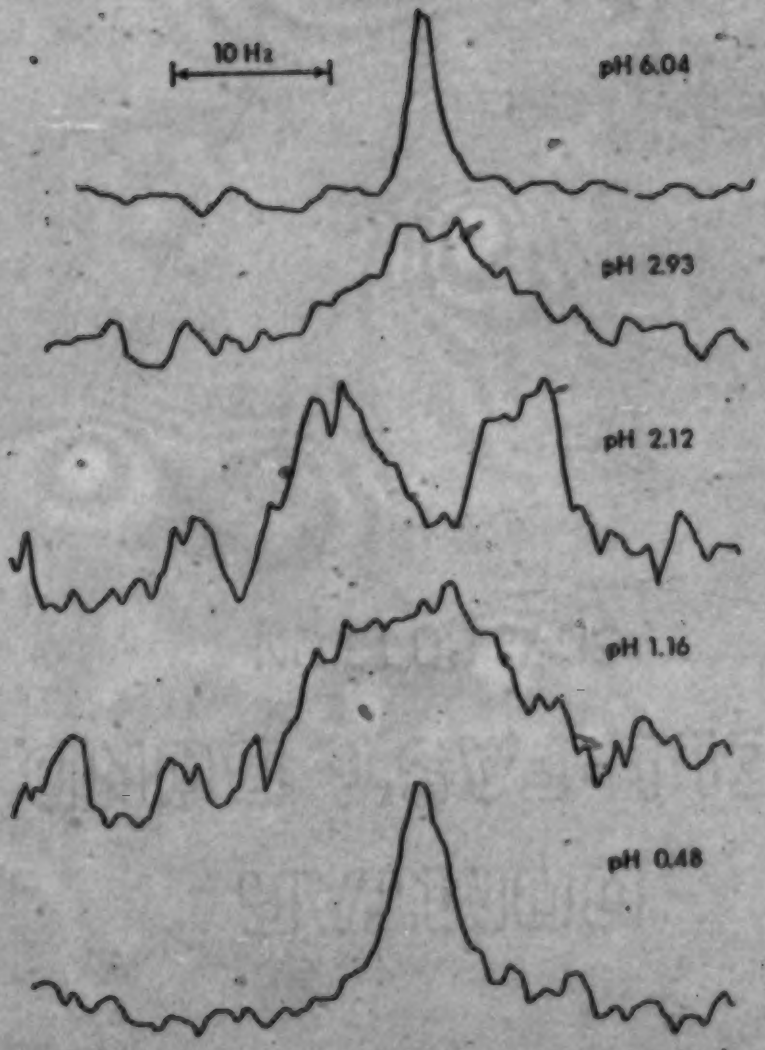


Figure 29: CYS-C_α region of the carbon-13 magnetic resonance spectra of a solution containing 0.165 M methylmercury-glutathione and 0.165 M glutathione as a function of pH. 25°C.

Table X. NMR Kinetic Data for the Methylmercury-glutathione System [a]

pH	[complexed glutathione] M	[free glutathione] M	$\frac{1}{\tau_c}$ [b] sec^{-1}
2.93	0.165	0.165	100
2.52	0.165	0.165	33
2.32	0.165	0.165	25
2.12	0.165	0.165	17
1.98	0.165	0.165	20
1.55	0.165	0.165	33
1.16	0.165	0.165	50
1.03	0.165	0.165	40
0.90	0.165	0.165	50
0.75	0.165	0.165	200
0.65	0.165	0.165	200
0.48	0.165	0.165	240
2.93	0.28	0.28	67
2.54	0.28	0.28	50
2.32	0.28	0.28	50
2.14	0.28	0.28	40
1.93	0.28	0.28	33
1.45	0.28	0.28	40
1.06	0.28	0.28	50
0.95	0.28	0.28	50

(Table continued)

Table X continued

pH	[complexed glutathione]	[free glutathione]	$\frac{1}{\tau_c}$ [b]
	M	M	sec^{-1}
0.80	0.28	0.28	67
0.68	0.28	0.28	100
0.47	0.28	0.28	200

[a] 25°C

[b] Obtained by comparison of experimental and computer simulated spectra. Uncertainty in $1/\tau_c$ values is estimated to be ± 15%.

Possible reactions by which glutathione might exchange between the free and complexed forms for the conditions of Table X are:

$$CH_3HgSG \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CH_3Hg^+ + GS^- \qquad (65)$$

$$CH_3HgSG + H^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CH_3Hg^+ + GSH \qquad (66)$$

$$CH_3HgSG + OH^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} CH_3HgOH + GS^- \qquad (67)$$

$$CH_3HgSG + \overset{*}{G}S^- \underset{}{\overset{k_4}{\rightleftharpoons}} CH_3HgS\overset{*}{G} + GS^- \qquad (68)$$

$$CH_3HgSG + G\overset{*}{S}H \underset{}{\overset{k_5}{\rightleftharpoons}} CH_3Hg\overset{*}{S}G + GSH \qquad (69)$$

where GSH, GS^-, and CH_3HgSG represent sulfhydryl-protonated, sulhydryl-deprotonated and sulfhydryl-complexed glutathione. The rate of decrease in the concentration of the complex is given by Equation (70)

$$-\frac{d[C]}{dt} = k_1[C] + k_2[H^+][C] + k_3[OH^-][C] + k_4\alpha_S[F][C] + k_5\alpha_{SH}[F][C] \qquad (70)$$

where [F] and [C] are the concentrations of free and complexed glutathione and $\alpha_S = [GS^-]/[F]$ and $\alpha_{SH} = [GSH]/[F]$.

Division by [C] leads to the following equation for the inverse of the mean lifetime of the complex.

$$\frac{1}{\tau_c} = k_1 + k_2[H^+] + k_3[OH^-] + k_4\alpha_S[F] + k_5\alpha_{SH}[F] \quad (71)$$

The relative importance of exchange by the reaction represented by Equation (65) can be predicted. By assuming that the reaction of CH_3Hg^+ and GS^- in Equation (65) is diffusion-controlled and therefore the rate constant k_{-1} has a value of 10^{10} $M^{-1}sec^{-1}$, we predict that $k_1 = k_{-1}K_F = 10^{-6}$ sec^{-1}, using the formation constant reported by Simpson (20) for the methylmercury-glutathione complex. If the rate constant k_{-1} is less than diffusion-controlled, k_1 will be even less, and thus the reaction represented by Equation (65) is predicted to contribute a negligible amount to $1/\tau_c$. The reaction represented by Equation (67) can also be shown not to contribute significantly to the observed exchange, at least up to a pH of approximately 4. If the reaction of OH^- with CH_3HgSG is diffusion-controlled and has a rate constant of 10^{10} $M^{-1}sec^{-1}$, its contribution to $1/\tau_c$ would only be 0.01 sec^{-1} at pH 2 and 1 sec^{-1} at pH 4. It is likely, however, that k_3 is somewhat less than diffusion-controlled since Simpson reported a value of 1.6×10^4 $M^{-1}sec^{-1}$ for the rate constant for the displacement of CN^- from CH_3HgCH by OH^- (32) in which case the contribution

to $1/\tau_c$ from the reaction represented by Equation (67) will be even less.

The relative importance of the reactions represented by Equations (66), (68) and (69) can be predicted from their dependence on solution conditions. The spectra shown in Figure 29 and the more extensive data in Table X for the solution containing 0.165 M methylmercury-gluthathione and 0.165 M free glutathione indicate that the rate of exchange is pH dependent; the minimum rate being at pH 2.1. The larger exchange rate at pH values less than 2.1 indicates that exchange occurs by proton-assisted dissociation of the complex at these pH values, while the increase in exchange rate at pH greater than 2.1 is due to displacement of complexed glutathione by GS^-, whose concentration increases as the pH increases. If the rate of exchange by the pH dependent reactions represented by Equations (66) and (68) is negligible at pH 2.12, the value of $1/\tau_c$ at this pH is equal to $k_5\alpha_{SH}[F]$. The rate of exchange by the reaction represented by Equation (69) will be pH independent for the pH range of the data in Table X since α_{SH}, the fraction of glutathione present as GSH, is greater than 0.999 over the pH range 1-6. Therefore the contribution of the reaction represented by Equation (69) will be the same as that at pH 2.12. If, however, some exchange is also occurring by the reactions represented by Equations (66) and (68) at pH 2.12, the

contribution of the reaction represented by Equation (69) to the observed $1/\tau_c$ will be even less, indicating that the amount of exchange by the reaction represented by Equation (69) for the conditions given in Table X is likely to be small relative to that by Equations (66) and (68). Since this value of 17 sec^{-1} for $1/\tau_c$ at pH 2.12 represents the upper limit for $k_5\alpha_{SH}[F]$ and is small compared to the variation observed in $1/\tau_c$, we shall neglect the contribution of this reaction to $1/\tau_c$ in a preliminary analysis of the data. Equation (71) then reduces to

$$\frac{1}{\tau_c} = k_2[H^+] + k_4\alpha_S[F] \qquad (72)$$

The exchange-broadened resonances of a solution containing 0.56 M glutathione and 0.28 M methylmercury never separate into the resonances of the free and complexed forms, indicating that the exchange rate is concentration dependent. The concentration dependence is also indicated by line shape changes in mole ratio experiments.

Rate constants k_2 and k_4 were estimated from the data in Table X by a method of successive approximations. First, a value was calculated for k_2 from each of the lifetimes at pH less than 2 by using Equation (72) and assuming $k_2[H^+] >> k_4\alpha_S[F]$. This procedure yielded an average value of 1.27×10^3 for k_2. Then a value for k_4 was calculated from each of the lifetimes for pH greater

than 2.1 using Equation (72) and this average value for k_2. Table XI lists the results of calculations of a new value for k_2 using the mean value for k_4. The mean value of k_2 is found to be 600 $M^{-1}sec^{-1}$ with a standard deviation of 200; this value was then used to recalculate k_4. The mean value of the results listed in Table XII for k_4 is 5.8×10^8 $M^{-1}sec^{-1}$ with a standard deviation of 1.9×10^8. From the value of k_2, k_{-2} is calculated as 5.1×10^9 $M^{-1}sec^{-1}$ using the relationship

$$k_{-2} = k_2 K_{SH} K_F \qquad (73)$$

where K_{SH} is the microscopic acid dissociation constant (73) for the sulfhydryl group and K_F is the formation constant (20).

In the calculation of the rate constants it was assumed that exchange by the reaction represented by Equation (69) was negligible. If however, the exchange at pH 2.12 is due solely to the reaction represented by Equation (69), the rate constants obtained by the above procedure would be $k_2 = 400 \pm 250$ $M^{-1}sec^{-1}$, $k_{-2} = 3.4 \times 10^9$ $M^{-1}sec^{-1}$, $k_4 = 2.5 \pm 1.5 \times 10^8$ $M^{-1}sec^{-1}$ and $k_5 = 110$ $M^{-1}sec^{-1}$. Since the actual contribution of the reaction represented by Equation (69) probably lies somewhere between the two extremes used in the above calculations, the two sets of rate constants can be considered as upper and lower limits on the actual rate constants.

Table XI. Calculated Values for k_2.

Expt. mole ratio	pH	k_2 $M^{-1}sec^{-1}$
0.3/0.15	0.48	740
0.3/0.15	0.65	890
0.3/0.15	0.72	1000
0.3/0.15	0.90	400
0.3/0.15	1.03	430
0.3/0.15	1.16	720
0.5/0.25	0.47	590
0.5/0.25	0.68	480
0.5/0.25	0.80	420
0.5/0.25	0.95	450
0.5/0.25	1.06	570

Table XII. Calculated Values for k_4.

Experiment	pH	$\alpha_{123}[F]$	$\frac{1}{\tau_c}$ sec^{-1}	k_4 $M^{-1}sec^{-1}$
0.3/0.15	3.42	4.67×10^{-7}	100	2.1×10^{8}
0.3/0.15	2.93	1.52×10^{-7}	100	6.5×10^{8}
0.3/0.15	2.52	5.88×10^{-8}	33	5.4×10^{8}
0.3/0.15	2.32	3.71×10^{-8}	25	5.9×10^{8}
0.3/0.15	2.12	2.35×10^{-8}	16.7	5.1×10^{8}
0.5/0.25	2.93	2.52×10^{-7}	66.7	2.6×10^{8}
0.5/0.25	2.54	1.03×10^{-7}	50	4.7×10^{8}
0.5/0.25	2.32	6.2×10^{-8}	50	7.6×10^{8}
0.5/0.25	2.14	4.1×10^{-8}	40	8.8×10^{8}

C. Discussion

For most methylmercury systems, the observed NMR spectra indicate that ligand and methylmercury exchange is fast on the NMR timescale. As mentioned in the introduction to this chapter, this was somewhat surprising considering the stability of some of the complexes formed. These observations however can be explained in terms of two aspects of the complexation chemistry of methylmercury as demonstrated by the kinetic results for the two systems studied in this chapter.

Firstly, methylmercury has a tendency to form polynuclear complexes. Reactions of the following type occur

$$CH_3HgX + CH_3Hg^+ \rightleftharpoons (CH_3Hg)_2X^+ \qquad (74)$$

which have the effect of labilizing the CH_3Hg-X bond.

In the methylmercury-methylamine system, exchange was slow enough to be observable on the NMR timescale because complexes of the type $(CH_3Hg)_2X$ cannot be formed. However, as shown in the previous chapter, a second methylmercury can bind to a methylmercury-complexed sulfhydryl group which apparently labilizes the sulfhydryl-complexed methylmercury and results in fast, exchange-averaged spectra for solutions having a methylmercury to glutathione ratio greater than 1.

Secondly, methylmercury complexes can react

with excess ligand according to the following reaction

$$CH_3HgX + X^* \rightleftarrows CH_3HgX^* + X \qquad (75)$$

These two types of reactions have the effect of causing exchange to be very rapid whenever there is even a slight excess of methylmercury or of ligand.

In the case of methylmercury-glutathione, fast exchange is observed for both proton and carbon-13 resonances except when excess glutathione is present. Results presented in this chapter show that reactions of this ligand displacement type are the predominant pathway for exchange of glutathione between free and complexed forms. Above pH 6, exchange is rapid. Below this pH, the concentration of sulfhydryl deprotonated ligand is small and the exchange by this pathway is less. At pH less than 2, exchange is rapid again due to a proton-assisted dissociation of the complex. In Chapter IV, results were presented which suggest the existence of a protonated-methylmercury-complexed sulfhydryl which possibly is an intermediate in the proton-assisted dissociation of the complex.

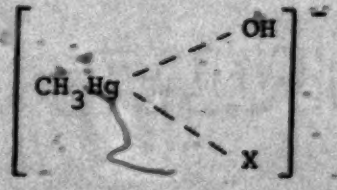
These conclusions are significantly different from those of Simpson, Hopkins and Hague (28) who studied the related ligand N-acetyl-L-cysteine. They concluded from PMR measurements of methylmercury-N-acetyl-L-cysteine solutions that exchange between the "free" and complexed

forms is by a pathway involving first order dissociation of the methylmercury complex. Their conclusions are questionable however, since the rate constant for the formation of the complex is predicted from their dissociation rate data to be six to seven orders of magnitude larger than diffusion-controlled bimolecular rate constants.

Eigen, Geier and Kruse (4) have studied ligand displacement reactions of the type

$$CH_3HgOH + X^- \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} CH_3HgX + OH^- \qquad (76)$$

where X is a halide ion. They have shown that k_{12} is strongly dependent on the identity of X, while k_{21} is reasonably independent of X and they have proposed that the reaction proceeds by an associative mechanism involving an intermediate of the type

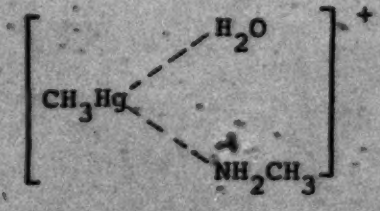


The formation constant of CH_3HgOH is larger than those of the halide complexes, suggesting that, once such an intermediate forms, the tendency for X^- to dissociate from the intermediate will be larger than for OH^-. In support of this (a) $k_{12} < k_{21}$ for the halides (b) both K_F and k_{12} increase in the order $Cl^- < Br^- < I^-$, and

(c) when X is CN^- which forms a methylmercury complex having log K_F = 14.1, compared to log K_F = 9.37 for CH_3HgOH, k_{21} is less than k_{12} and is approximately 5 orders of magnitude less than when X is a halide ion.

The mechanism of reactions of the type represented by Equation (75) presumably proceeds through an intermediate analogous to that proposed by Eigen, Geier and Kruse for methylmercuric hydroxide reactions. If this is the case, there is an equal probability that once the intermediate forms, it will dissociate to products or reactants. Thus the rate of ligand exchange is predicted to be one half the rate of formation of the intermediate, which is expected to be determined by the rate of diffusional encounter or to be slightly less than diffusion-controlled rate constants due to steric effects.

It is not known if complex formation reactions involving $CH_3HgOH_2^+$ proceed by an associative or dissociative mechanism. In the formation of the methylmercury-methylamine complex from $CH_3HgOH_2^+$ and CH_3NH_2, which has a rate constant of 3×10^9 sec^{-1}, the possible mechanisms are: for an associative mechanism, the reaction would proceed through an intermediate such as

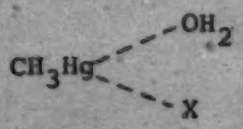


while for a dissociative mechanism the reaction would proceed through the following two steps

$$CH_3HgOH_2^+ \rightleftarrows CH_3Hg^+ + H_2O \qquad (77)$$

$$CH_3Hg^+ + CH_3NH_2 \rightleftarrows CH_3HgNH_2CH_3^+ \qquad (78)$$

with the first being the rate-determining step. The results presented in this thesis for the reaction of methylamine with $CH_3HgOH_2^+$ and other results for $CH_3HgOH_2^+$ reported in the literature do not allow one to distinguish between these 2 possible mechanisms. With other metal ions, the mechanism has been identified by studying the dependence of the rate of exchange on the identity of the ligand. For example, if the rate of a complexation reaction for an aquated metal ion $M(H_2O)_6^{++}$ does not depend on X, the mechanism is assumed to be dissociative with the rate of water loss being the rate-determining step. On the other hand, if the rate depends on X, an associative mechanism is assumed. The data presently available for complexation reactions of $CH_3HgOH_2^+$ do not allow such an analysis for the $CH_3HgOH_2^+$ reactions because, for the systems studied to date, the formation constants of the CH_3HgX complexes are so large that even if the reactions are associative and proceed through intermediates such as



, the rate of the reaction will not depend

on the nature of X since dissociation of water from the intermediate will be faster than dissociation of X. One way to resolve this problem might be to study ligands whose affinity for the methylmercury cation is similar to that of water. Ligands that might be suitable are those with the thioether functional group.

The results reported in this chapter for the ligand exchange kinetics in the methylmercury-glutathione system have implications as to the lability of methylmercury in biological systems. In biological systems, methylmercury is bound by sulfhydryl-containing proteins. In the sulfhydryl-containing ligands studied in this thesis the methylmercury binding was very labile. This was shown to be due to the presence of excess methylmercury or excess sulfhydryl ligand. When methylmercury is bound by a protein it is unlikely that excess methylmercury is present or that there would be another nearby sulfhydryl group in a position suitable for binding. Thus these results suggest that the methylmercury bound by a protein is probably quite inert kinetically.

BIBLIOGRAPHY

1. For a recent review see D. Seyferth, J. Organomet. Chem., 62, 33 (1973).
2. G. Schwarzenbach and N. Schellenberg, Helv. Chim. Acta, 48, 28 (1965).
3. F. Basolo and R.G. Pearson "Mechanisms of Inorganic Reactions", Wiley, New York, 1958, p. 23.
4. M. Eigen, G. Geier and W. Kruse, "Essays in Coordination Chemistry", Experimentia (Birhäuser Verlag, Basel, 1965) Suppl. 9 p. 164.
5. M. Eigen, Pure Appl. Chem., 6, 97 (1963).
6. W.L. Hughes, Jr., Cold Spring Harbor Symposia, XIV, 79 (1950).
7. T.A. Beerman, and J. Lebowitz, J. Mol. Biol., 79, 451 (1973).
8. L.T. Kurland, S.N. Faro and H. Siedler, World Neurol., 1, 370 (1960).
9. J.M. Wood, F. Scott Kennedy, and C.G. Rosen, Nature, 220, 173 (1968).
10. H.A.O. Hill, J.M. Pratt, S. Ridsdale, F.R. Williams and P.J. Williams, Chem. Commun., 341 (1970).
11. K. Kohei and F. Masahiko, Toxicology, 1, 43 (1973).
12. T. Takeuchi, Pathology of Minimata disease, Study Group of Minimata Disease, Kumamoto University, Japan, August 1968 p. 141-252.
13. T.W. Clarkson, CRC Crit. Rev. in Toxicology, 1, 203 (1971-72).

14. T.D. Waugh, H.T. Walton and J.A. Laswick, J. Phys. Chem., 59, 395 (1955).

15. S. Mansy, T.E. Wood, J.C. Sprowles and R.S. Tobias, J. Amer. Chem. Soc., 96, 1762 (1964).

16. J.H.S. Green, Spectrochimica Acta, 24A, 863 (1968).

17. D. Grdenic and T. Zado, J. Chem. Soc., 521 (1962).

18. F.A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry", John Wiley and Sons, New York, 1966 p. 620.

19. P.L. Goggin and L.A. Woodward, Trans. Farad. Soc., 62, 1423 (1966).

20. P.B. Simpson, J. Amer. Chem. Soc., 83, 4711 (1961).

21. L.T. Sytsma and R.J. Kline, J. Organomet. Chem., 54, 15 (1973).

22. T.S. Wong, P.C. Chieh, and A.J. Carty. Can. J. Chem., 51, 2597 (1973).

23. R.B. Simpson, J. Amer. Chem. Soc., 86, 2059 (1964).

24. D.W. Gruenwedel and N. Davidson, J. Mol. Biol., 21, 129 (1966).

25. P.L. Goggin and L.A. Woodward, Trans. Farad. Soc., 58, 1495 (1962).

26. R.W. Chen, H.E. Ganther, and W.G. Hoekstra, Biochem. Biophys. Res. Commun., 51, 383 (1973).

27. B.J. Fuhr and D.L. Rabenstein, J. Amer. Chem. Soc., 95, 6944 (1973).

28. P.G. Simpson, T.E. Hopkins, and R. Hague, J. Phys. Chem., 77, 2282 (1973).

29. Y.S. Wong, P.C. Chieh and A.J. Carty, Chem. Commun., 741 (1973).

30. J.V. Halton, W.G. Schneider and W. Siebrand, J. Chem. Phys., 39, 1330 (1963).

31. D.N. Ford, P.R. Wells and P.C. Lauterbiar, Chem. Commun., 616 (1967).

32. N.S. Ham, E.A. Jeffery, T. Mole and S.N. Stuart, Chem. Commun., 254 (1967).

33. R.B. Simpson, J. Chem Phys., 46, 4775 (1967).

34. D.F. Evans, P.M. Ridout and I. Wharf, J. Chem. Soc. (A)., 2127 (1968).

35. S. Libich and D.L. Rabenstein, Anal. Chem., 45, 118 (1973).

36. L.A. Fedorov, V.N. Kalinin, K.G. Gasanov and L.J. Zakharkin, Zhurnal Organicheskoi Khinaii, 7, 432 (1971).

37. P.L. Goggin, R.J. Goodfellow, S.R. Haddock and J.G. Cary, J. Chem. Soc. Dalton, 647 (1972).

38. K.P. Butin, I.P. Beletskaya and O.A. Reutov, J. Organomet. Chem., 64, 323 (1974).

39. W.J. Blaedel and V.W. Meloche, "Elementary Quantitative Analysis", Harper and Row, New York, 1963, p. 794.

40. L.A. van Geet, Anal. Chem., 42, 679 (1970).

41. J.W. Emsley, J. Feeney and L.H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance". Vol. 1, Pergamon Press, Oxford, 1965, pp. 481-488.

42. J.A. Pople, W.G. Schneider and H.J. Bernstein, "High Resolution Nuclear Magnetic Resonance", McGraw-Hill Book Co., New York, 1959, pp. 218-225.

43. C.S. Johnson, Jr., in "Advances in Magnetic Resonance", Vol. 1, J.S. Waugh, Ed., Academic Press, New York, 1965, pp. 33-102.

44. R.M. Lynden-Bell in "Progress in Nuclear Magnetic Resonance Spectroscopy", Vol. 2, J.W. Emsley, J. Feeney and L.H. Sutcliffe, Eds., Pergamon Press Ltd., Oxford, 1967, pp. 163-204.

45. G. Binsch, in "Topics in Stereochemistry", Vol. 3, E.L. Eliel and N.L. Alliger, Eds., John Wiley and Sons, New York, 1968.

46. F. Bloch, Phys. Rev., 70, 460 (1946).

47. H.S. Gutowsky, D.W. McCall and C.P. Slichter, J. Chem. Phys., 21, 279 (1953).

48. H.S. Gutowsky and C.H. Holm, ibid., 25, 1228 (1956).

49. H.M. McConnell, ibid., 28, 430 (1958).

50. Reference 42. p. 221.

51. J.H.R. Clarke and L.A. Woodward, Trans. Faraday Soc., 62, 3022 (1966).

52. P.L. Goggin and L.A. Woodward, Trans. Faraday Soc., 56, 1591 (1960).

53. P.L. Goggin and L.A. Woodward, Trans. Faraday Soc., 58, 1495 (1962).

54. J. Lorbeth and F. Weller, J. Organomet. Chem., 32, 145 (1971).

55. J.H. Clarke and L.A. Woodward, Trans. Faraday Soc., 64, 1041 (1971).

56. D.L. Rabenstein, C.A. Evans, M.C. Tourangeau and M.T. Fairhurst, Anal. Chem., in press.

57. J.H. Clarke and L.A. Woodward, Spectrochim. Acta, 23A, 2077 (1967).

58. D.L. Rabenstein, R. Ozubko, S. Libich, C.A. Evans and M.T. Fairhurst, J. Coord. Chem., 3, 263 (1974).

59. D.L. Rabenstein and M.T. Fairhurst, J. Amer. Chem. Soc., in press.

60. M.T. Fairhurst and D.L. Rabenstein, Inorg. Chem., in press.

61. L.G. Sillen and A.E. Martell, "Stability Constants of Metal Ion Complexes", The Chemical Society, London 1964.

62. H.A. Laitinen, "Chemical Analysis", McGraw-Hill Book Company, New York, 1960, pp. 35-36.

63. D.F.S. Natusch and L.J. Porter, Chem. Commun., 596 (1970).

64. D.F.S. Natusch and L.J. Porter, J. Chem. Soc. A, 2527 (1971).

65. E. Grunwald, A. Loewenstein and S. Meiboom, J. Chem. Phys., 27, 641 (1957).

66. M. Christl and J.D. Roberts, J. Amer. Chem. Soc., 94, 4565 (1972).

67. N.C. Li and R.A. Manning, ibid., 77, 5225 (1955).

68. G.R. Lenz and A.E. Martell, Biochemistry, 3, 745 (1964).

69. C.A. McAuliffe, J.V. Quagliano and L.M. Vallarino, Inorg. Chem., 5, 1996 (1966).

70. Y.S. Wong, N. Taylor, P. Chieh and A. Carty, Chem. Commun., 625 (1974).

71. R. Sheffold, Helv. Chim. Acta, 52, 56 (1969).

72. I. Amdur and G.G. Hammes, "Chemical Kinetics: Principles and Selected Topics", McGraw-Hill Book Company, New York, 1966, pp. 60-64.

73. D.L. Rabenstein, J. Amer. Chem. Soc., 95, 2797 (1973).

APPENDIX I

Calculation of Concentrations of Species Present in Methylmercury-Methylamine System

The following derivation is for the calculation of the concentrations of the species present in solutions having equal total concentrations of methylmercury and methylamine, i.e.,

$$(CH_3Hg)_{TOTAL} = (MeAm)_{TOTAL} = L_T \qquad (1)$$

The formation constant, K_F, of the methylamine complex is known from previous work (58): $\log K_F = 7.59$.

$$K_F = \frac{[\text{complex}]}{[CH_3NH_2][CH_3Hg(II)]} \qquad (2)$$

Letting P_c equal the fraction of methylmercury which is complexed, Equation (3) follows from Equations (1) and (2).

$$K_F = \frac{(P_c L_T)}{(\alpha_B(1-P_c)L_T)(\beta(1-P_c)L_T)} \qquad (3)$$

where $$\alpha_B = \frac{[CH_3NH_2]}{[CH_3NH_2] + [CH_3NH_3^+]} \qquad (4)$$

Expressing the concentrations in terms of the acid dissociation constant leads to

$$\alpha_B = \frac{K_A}{[H^+] + K_A} \qquad (5)$$

In Equation (3), β represents the fraction of methyl-

mercury in the cation form.

The free methylmercury is distributed between three forms: $CH_3Hg(II)$, CH_3HgOH, and $(CH_3Hg)_2OH^+$. The trimer, $(CH_3Hg)_3O^+$, is ignored since calculations show that only a very small amount is present at these concentrations of methylmercury (see Chapter III). Because of the unsymmetrical nature of the equilibria relating the concentrations of the three forms of methylmercury, the concentration of methylmercury in the cation form depends on the concentration of free methylmercury. Thus P_c and β must be determined simultaneously since the value for one depends on the value obtained for the other.

Equation (3) may be rewritten as

$$K_F = \frac{P_c L_T}{(L_T^2 \alpha_B \beta - P_c L_T^2 \alpha_B \beta - P_c L_T^2 \alpha_B \beta + P_c^2 L_T^2 \alpha_B \beta)} \quad (6)$$

which rearranges to

$$(K_F L_T^2 \alpha_B \beta) P_c^2 - (2K_F L_T^2 \alpha_B \beta + L_T) P_c + K_F L_T^2 \alpha_B \beta = 0 \quad (7)$$

Equation (7) is a quadratic of the form $ax^2 + bx + c = 0$

where $a = K_F L_T^2 \alpha_B \beta$

$b = -(2K_F L_T^2 \alpha_B \beta + L_T)$

$c = K_F L_T^2 \alpha_B \beta$

Thus

$$P_c = \frac{-b - \sqrt{b^2 - 4ac}}{2a} \quad (8)$$

Only the negative root of Equation (7) is physically meaningful. The method of solving for P_c was to make an initial guess at β, the fraction of methylmercury in the cation form. A suitable initial guess for β is β = 1 which is equivalent to assuming that all the methylmercury is in the cation form. P_c is then calculated using Equation (8). Then using a concentration of free methylmercury equal to $(1 - P_c)L_T$, a new value of β was obtained using Equation (38) Chapter IV. This new value of β was then used to calculate a new value for P_c. The iterative procedure was repeated until P_c and β both converged.

Once P_c and β are known the concentrations of the various forms of methylmercury can be calculated using the following equations:

$$[CH_3Hg(II)] = \beta\,(1-P_c)L_T \qquad (9)$$

$$[CH_3HgOH] = 10^{-4.70}([CH_3Hg(II)])([OH^-]) \qquad (10)$$

$$2[(CH_3Hg)_2OH^+] = L_T - [complex] - [CH_3Hg(II)] - [CH_3HgOH] \qquad (11)$$

From the concentrations of the three forms of free methylmercury and their chemical shifts, the exchange averaged shift of the free methylmercury was calculated using Equation (12)

$$\delta_F = P_{CATION}\delta_{CATION} + P_{DIMER}\delta_{DIMER} + P_{BASE}\delta_{BASE} \qquad (12)$$

where

$$P_{CATION} = \frac{[CH_3Hg(II)]}{[CH_3Hg(II)] + [CH_3HgOH] + 2[(CH_3Hg)_2OH^+]} \quad (13)$$

$$P_{DIMER} = \frac{2[(CH_3Hg)_2OH^+]}{[CH_3Hg(II)] + [CH_3HgOH] + 2[(CH_3Hg)_2OH^+]} \quad (14)$$

$$P_{BASE} = \frac{[CH_3HgOH]}{[CH_3Hg(II)] + [CH_3HgOH] + 2[(CH_3Hg)_2OH^+]} \quad (15)$$

Using P_c and δ_F, the lifetime of the complexed species was calculated as described in Chapter V.

PART 2

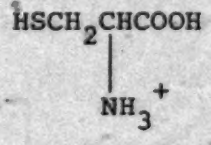
THE ACID-BASE CHEMISTRY OF AMINO ACIDS

CHAPTER VI

INTRODUCTION

The acid-base chemistry of amino acids has been a subject of interest for some time; acid dissociation constants have been used in the identification of amino acids and in characterizing their solution chemistry.

The acid-base chemistry of amino acids is quantitatively described by stepwise acid dissociation constants; for example, for a triprotic amino acid such as fully-protonated cysteine,



H_3L^+, acid dissociation occurs in the following stepwise fashion and is described by acid dissociation constants K_1, K_2 and K_3.

$$H_3L^+ \rightleftarrows H^+ + H_2L \qquad (1)$$

$$K_1 = \frac{[H^+][H_2L]}{[H_3L^+]} \qquad (2)$$

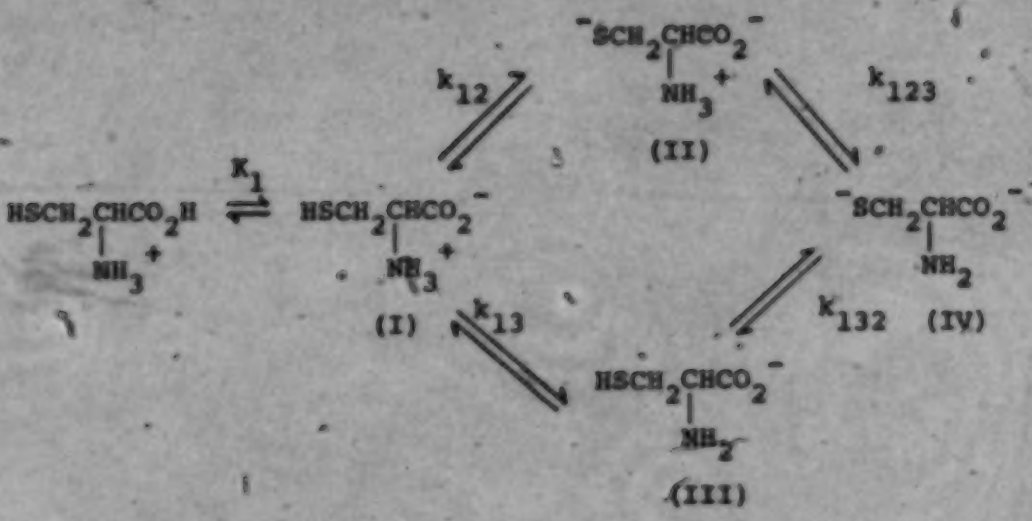
$$H_2L \rightleftarrows H^+ + HL^- \qquad (3)$$

$$K_2 = \frac{[H^+][HL^-]}{[H_2L]} \qquad (4)$$

$$HL^- \rightleftharpoons H^+ + L^{2-} \qquad (5)$$

$$K_3 = \frac{[H^+][L^{2-}]}{[HL^-]} \qquad (6)$$

The most common method for the evaluation of acid dissociation constants is based on pH titrations. By comparison of the K_A values of cysteine with those of carboxylic acids, amines and sulfhydryl compounds it has been established that K_1 describes the deprotonation of the carboxylic acid group. The assignment of K_2 and K_3 to the remaining acidic groups, however, is not so straightforward. The pK_A for the sulfhydryl group of ethylmercaptan is reported to be 10.50 (1) while that for the amino group of methylamine is 10.72 (2). Thus the acid strength of the ammonium group is of the same order of magnitude as that of the sulfhydryl group. Consequently, at the molecular level, acid dissociation of deprotonated cysteine can occur by two different pathways, which are shown below in the microscopic acid dissociation scheme.



The microscopic acid dissociation constants, defined by Equations (7) - (10), are generally represented by lower case k's (3). The acid dissociation step which is described by a given microscopic constant is specified first by the last number in the subscript, which denotes the position of the proton involved in the reaction under consideration, and second by the whole set of preceding numbers in the subscript, which, regardless of order, denote the other positions from which protons have already been removed. In cysteine, the carboxyl, sulfhydryl and ammonium groups are denoted by numbers 1, 2 and 3 respectively.

$$k_{12} = \frac{[H^+][II]}{[I]} \qquad (7)$$

$$k_{13} = \frac{[H^+][III]}{[I]} \qquad (8)$$

$$k_{123} = \frac{[H^+][IV)}{[II]} \qquad (9)$$

$$k_{132} = \frac{[H^+][IV]}{[III]} \qquad (10)$$

It is not possible to determine the microscopic acid dissociation constants of cysteine from its pH titration curve alone. By pH titration the concentration of the monoprotonated form, which is the sum of the concentrations of the two individual monoprotonated forms, can be determined but not the concentrations of the two monoprotonated isomers.

The macroscopic acid dissociation constants can be shown to be composites of the microscopic constants.

$$K_2 = \frac{[H^+]([II] + [III])}{(I)} \qquad (11)$$

$$K_2 = k_{12} + k_{13} \qquad (12)$$

$$K_3 = \frac{[H^+][IV]}{([II] + [III])} \qquad (13)$$

$$\frac{1}{K_3} = \frac{1}{k_{123}} + \frac{1}{k_{132}} \qquad (14)$$

Several pathways are available for the deprotonation of several of the common amino acids. A few examples are lysine, aspartic acid, glutamic acid,

penicillamine, tyrosine, and homocysteine. Many peptides also contain several functional groups of similar acid strength and thus can be characterized in this way. For example, any peptide containing a lysine residue has at least two amino groups, the N-terminal amino group and the lysine-6-amino group. Peptides containing glutamic or aspartic acid residues contain a terminal carboxylic acid plus the carboxylic acid group in the glutamyl or aspartyl side chain. Glutathione is an example of such a peptide. A knowledge of the microscopic dissociation constants for such peptides may be useful when studying the chemistry of these molecules. For example, in considering the coordination chemistry of a molecule such as cysteine, if one of the two monoprotonated forms specifically forms a metal complex, the microscopic constants must be known in order to determine the formation constant of the complex.

In general, most compounds have not been characterized in terms of their microscopic dissociation constants because the required information is not available from pH titrations. To determine the microscopic dissociation constants a method of monitoring dissociation at specific sites is needed. The various methods which have been used to determine microscopic ionization constants will now be described using cysteine as an example since it has been studied the most extensively.

In the cysteine system, a popular approach has been the use of model compounds. Ryklan and Schmidt (4) assumed that the effect of an S-alkyl group on the deprotonation of a neighboring group would be approximately the same as that of an un-ionized SH group. They determined the pK_A for the ammonium group in S-ethylcysteine to be 8.60 at 25°, which they considered to be equal to the microscopic constant pk_{13} for cysteine itself. They then used this value in conjunction with the macroscopic constants to determine values for the remaining microscopic constants. [Equations (12) and (14)]. Their results are listed in Table XIII.

Grafius and Neilands (5) determined the microscopic dissociation constants in a similar way using the pK_A determined for the sulfhydryl group of cysteine betaine, which contains a $-N(CH_3)_3^+$ group instead of the $-NH_3^+$ group of cysteine. These workers assumed the trimethyl ammonium group resembles the NH_3^+ group in its effect on the deprotonation of the sulfhydryl group and thus that the pK_{A2} of cysteine betaine equals pk_{12} of cysteine.

A more direct approach to the problem was made by Benesch and Benesch (6), who used the ultraviolet absorption spectrum of the ionized -SH group. From the ultraviolet data, the fraction of the cysteine sulfhydryl groups in the deprotonated form could be determined

Table XIII. Microscopic Acid Dissociation Constants of Cysteine.

pk_{12}	pk_{13}	pk_{123}	pk_{132}	method	ref.
8.66	8.60	10.45	10.51	pH titration using S-ethylcysteine as model compound	(4)
8.65	8.75	-	-	pH titration using S-methylcysteine and cysteine betaine as model compounds	(5)
8.53	8.86	10.36	10.03	ultraviolet absorption spectroscopy	(6)
8.50	8.85	10.35	10.00	Raman spectroscopy	(7)
8.44	8.31	9.58	9.73	calorimetric methods	(8)
8.54	8.86	10.53	10.21	ultraviolet spectroscopy	(9)

from the ratio of the absorption at the given pH value to the absorption when the -SH group is completely dissociated. Using this fraction along with the macroscopic dissociation constants, the microscopic constants were determined. Their results are listed in Table XIII. The acid-base chemistry of cysteine has been studied by a number of other workers using the ultraviolet absorption method developed by Benesch and Benesch. Some of their results are also listed in Table XIII.

Elson and Edsall (7) determined the microscopic constants by monitoring the deprotonation of the sulfhydryl group by Raman spectroscopy. The results they obtained at relatively high ionic strengths are close to those obtained by Benesch and Benesch who worked at an ionic strength near 0.2.

Wrathall, Izatt and Christensen (8) described a calorimetric method for determining microscopic acid dissociation constants. The method is based on the fact that the ΔH values for proton dissociation from the NH_3^+ group of S-methylcysteine and the -SH group of mercaptoacetic acid differ by approximately 4 kcal/mole. The values they reported varied greatly with ionic strength; their results at an ionic strength of 1 are reported in Table XIII. This method has been criticized by Wilson and Martin (10), who point out that the calorimetric method requires that heats for both sulfhydryl de-

protonations and, separately, both ammonium deprotonations be equal and hence independent of the ionic state of the remainder of the molecule.

Most of the experiments described above for the determination of the microscopic acid dissociation constants of cysteine were possible because of the special properties of the sulfhydryl group. Thus these methods are not applicable to many amino acids and peptides, for example amino acids or peptides containing two amino groups or two carboxyl groups. There is no direct method available for systems of these types.

Since the chemical shift, δ, of a magnetically-active nucleus is sensitive to the state of protonation of nearby acidic groups, nuclear magnetic resonance spectroscopy might be a general method for determining microscopic acid dissociation constants. Grunwald, Loewenstein and Meiboom (11) found that, in solutions of methylamine and methylamine hydrochloride, the chemical shift of the methyl protons is a linear function of the concentration ratio of acid to base plus acid and thus, that it is possible to obtain acid dissociation constants by using the chemical shift to determine the fractions of protonated and deprotonated forms.

Proton magnetic resonance spectroscopy has been used by several groups of workers to measure microscopic dissociation constants. Lowenstein and Roberts (12)

studied the ionization of citric acid using the shift of the methylene protons as a measure of the state of protonation. To determine the effect of the central carboxyl deprotonation on the methylene shift they used the citric acid symmetrical monomethyl ester. Thus they assumed the methylated carboxyl group behaves with regard to the chemical shift as if it were a non-ionized carboxyl group. Their results have been criticized by Martin (13).

Rigler et. al. (14) investigated the microscopic acid-base equilibria of tetracycline using proton magnetic resonance techniques. They used a quaternary methyl-ammonium derivative to assist in separating the effects of deprotonation of various grups on the chemical shift.

More recently, Walters and Leyden (15) have applied proton magnetic resonance spectroscopy to the problem of microscopic constant determination of cysteine. Since the shift of the protons is affected by both amino and sulfhydryl deprotonations they found it necessary to use a model compound, S-methylcysteine, to assign shifts to the intermediate monoprotonated species. The validity of such a procedure is questionable and is discussed further in Chapter VIII. Their results are in close agreement with the calorimetric results (8) but disagree with the results obtained by other methods (6,7).

In all examples of the application of NMR to microscopic constant determination discussed thus far,

the chemical shift was affected by more than one deprotonation reaction occurring simultaneously. Model compounds were used in each case to separate the effects of these deprotonation reactions so that chemical shift values could be obtained for the intermediate species. The situation is simplified somewhat when the chemical shift is affected only by deprotonation at a single site. There are a few examples of such studies in the literature. The acid-base chemistry of fully protonated glutathione and methylmercury-complexed glutathione has been studied by PMR (16). In glutathione, the simultaneously-deprotonating groups are well removed from each other, thus the chemical shift of a given proton reflects the fractional deprotonation of only the nearby acidic group. The microscopic constants were determined from the fractional deprotonation of each of the acidic groups as a function of pH.

Creyf, van Poucke, and Eeckhaut (17) used a similar method to obtain the microscopic acidity constants of deprotonated asymmetric N-methylsubstituted ethylenediamines and N-methylpiperazine. The shift of the methyl group is influenced only by changes in the protonation of the nitrogen atom adjacent to the methyl group.

These workers all used proton magnetic resonance techniques. Carbon-13 magnetic resonance is a

potentially more useful tool. In the proton-decoupled carbon-13 spectrum single lines are observed for each carbon atom making it possible to follow deprotonation at each site in the molecule. In addition the range of chemical shifts is much larger than that observed in proton spectra making carbon-13 chemical shifts more sensitive to the state of protonation. Thus, it was thought that carbon-13 magnetic resonance spectroscopy might be a useful probe for the study of the acid-base chemistry of amino acids. The results obtained from a study of the acid-base chemistry of cysteine and related molecules by carbon-13 NMR are the subject of Part 2 of this thesis.

CHAPTER VII

EXPERIMENTAL

A. Chemicals

D,L-penicillamine, 2-aminoethanethiol hydrochloride (Aldrich Chemical Company) and S-methylcysteine (Nutritional Biochemicals Corp.) were used as received. Cysteine (Nutritional Biochemicals Corp.) was recrystallized from water as the free base.

All other chemicals were reagent grade and were used without further purification.

B. Preparation of Solutions

In general all solutions were prepared as described in Chapter II Section C, with the following modification to minimize ionic strength variations from sample to sample. Initially the pH was adjusted to ~13 with potassium hydroxide or to ~1 with nitric acid. Nitric acid or potassium hydroxide was then added and samples were withdrawn at the appropriate pH values. Using this procedure the ionic strength of a 0.2 M solution of cysteine for example varied from 0.9 at pH 13 to 0.7 at pH 5.

C. pH Measurements

pH measurements were made as described in

Chapter II Section D.

D. Calculation of Hydrogen Ion Concentration

In Chapter VIII the acid dissociation constants are determined as concentration constants. To convert pH meter readings, which are related to hydrogen ion activities, to p_cH, the negative logarithm of the hydrogen ion concentration, the activity coefficient must be known. In order to calculate the activity coefficient for the hydrogen ion, the ionic strength must first be calculated using Equation (15).

$$\mu = \frac{1}{2} \Sigma C_i z_i^2 \qquad (15)$$

where μ represents the ionic strength of a solution containing i different ions of concentration C_i and charge z_i. In the solutions discussed in Chapter VIII the amount of ligand in the various charged forms was calculated assuming approximate values for the macroscopic constants. The concentrations of nitrate and potassium ions were calculated from the amounts of nitric acid and potassium hydroxide which were added to prepare the sample. The concentrations of hydrogen and hydroxyl ions were calculated from the solution pH.

Once the ionic strength was calculated, the activity coefficient for the hydrogen ion was found using

the Davies Equation (18)

$$-\frac{\log \gamma_i^2}{n^2} = \frac{0.511\ (\mu)^{1/2}}{1 + 1.5\ (\mu)^{1/2}} - 0.2\ (\mu) \qquad (16)$$

where γ_i is the activity coefficient of an ion in charge n. Using the meter reading as the negative logarithm of the activity of the hydrogen ion and the calculated activity coefficient, p_cH, the negative logarithm of the hydrogen ion concentration, was calculated.

E. Non-Linear Least Squares Program

In Chapter VIII the values for macroscopic dissociation constants were obtained from a non-linear least squares analysis of the chemical shift data. The non-linear least squares program used is based on the method described by Marquardt (19) and is available in the Chemistry Department of the University of Alberta as ENLLSQ.

F. Carbon-13 Magnetic Resonance Measurements

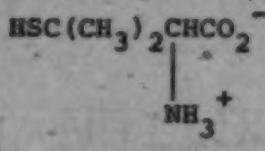
Carbon-13 magnetic resonance measurements were made as described in Chapter II Section F with the exception that the number of pulses was reduced to 2K.

CHAPTER VIII

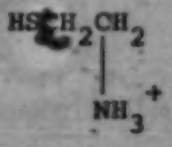
CARBON-13 NMR STUDY OF THE ACID-BASE CHEMISTRY OF CYSTEINE AND RELATED MOLECULES

A. Introduction

In this chapter, the application of carbon-13 NMR to the study of the acid-base chemistry of amino acids is investigated. The compounds chosen for this study are cysteine, which has been studied extensively by other techniques, penicillamine,



and 2-aminoethanethiol



First, the evaluation of macroscopic dissociation constants using carbon-13 chemical shift data will be described and then the attempts to evaluate microscopic dissociation constants will be discussed.

B. Results

1. Determination of Macroscopic Acid Dissociation Constants from Carbon-13 Chemical Shifts.

In this section, a method for the evaluation of overlapping macroscopic acid dissociation constants using carbon-13 chemical shift measurements is presented. The method is applied to the evaluation of macroscopic dissociation constants for the deprotonation of the amino and sulfhydryl groups of cysteine, penicillamine and 2-aminoethanethiol. The macroscopic dissociation constants for these deprotonation reactions will be described by K_2 and K_3 although in the case of 2-aminoethanethiol these dissociations really represent K_1 and K_2.

$$H_2L \rightleftharpoons HL + H \tag{17}$$

$$K_2 = \frac{[HL][H]}{[H_2L]} \tag{18}$$

$$HL \rightleftharpoons H + L \tag{19}$$

$$K_3 = \frac{[H][L]}{[HL]} \tag{20}$$

In Equations (17)-(20) charges have been omitted for simplicity.

Carbon-13 data for penicillamine, cysteine and 2-aminoethanethiol were collected over the pH range 4-13 where the amino and sulfhydryl deprotonations occur. This data is presented in Figures 30, 31 and 32. For cysteine and penicillamine, the assignment was done by

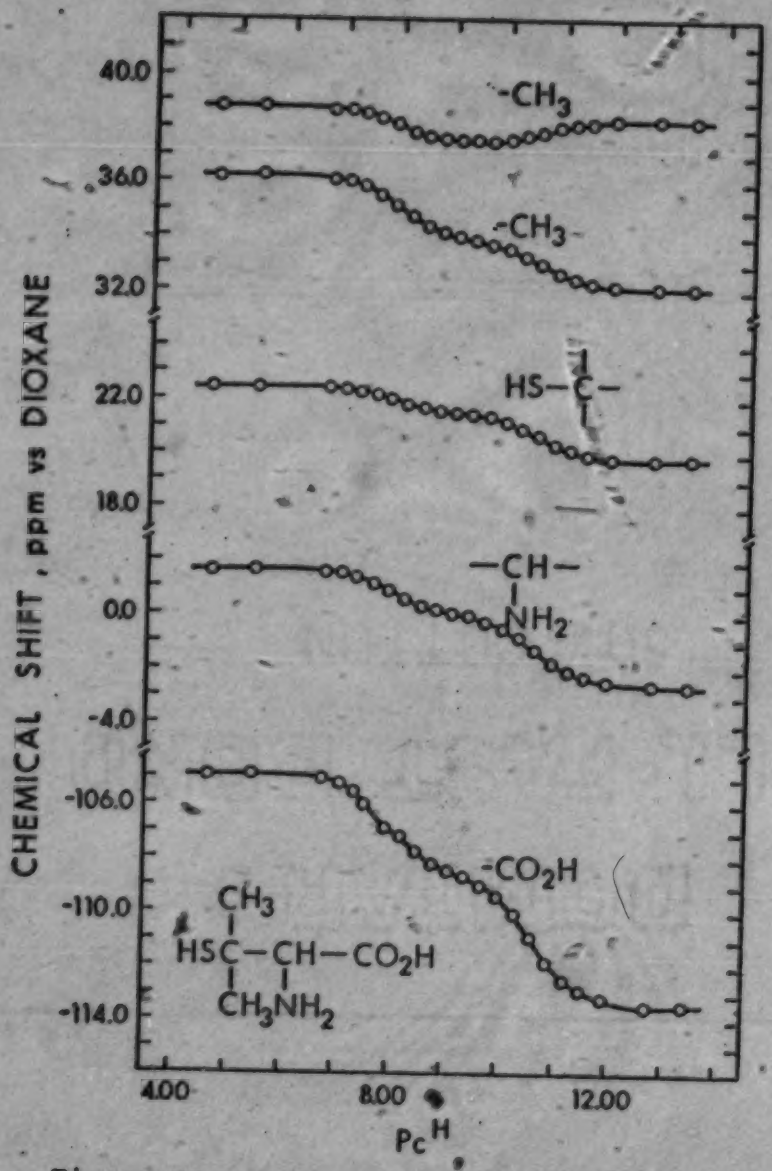


Figure 30: pH dependence of the carbon-13 chemical shifts of the five penicillamine carbon atoms in an aqueous solution containing 0.20 M penicillamine. 25°C.

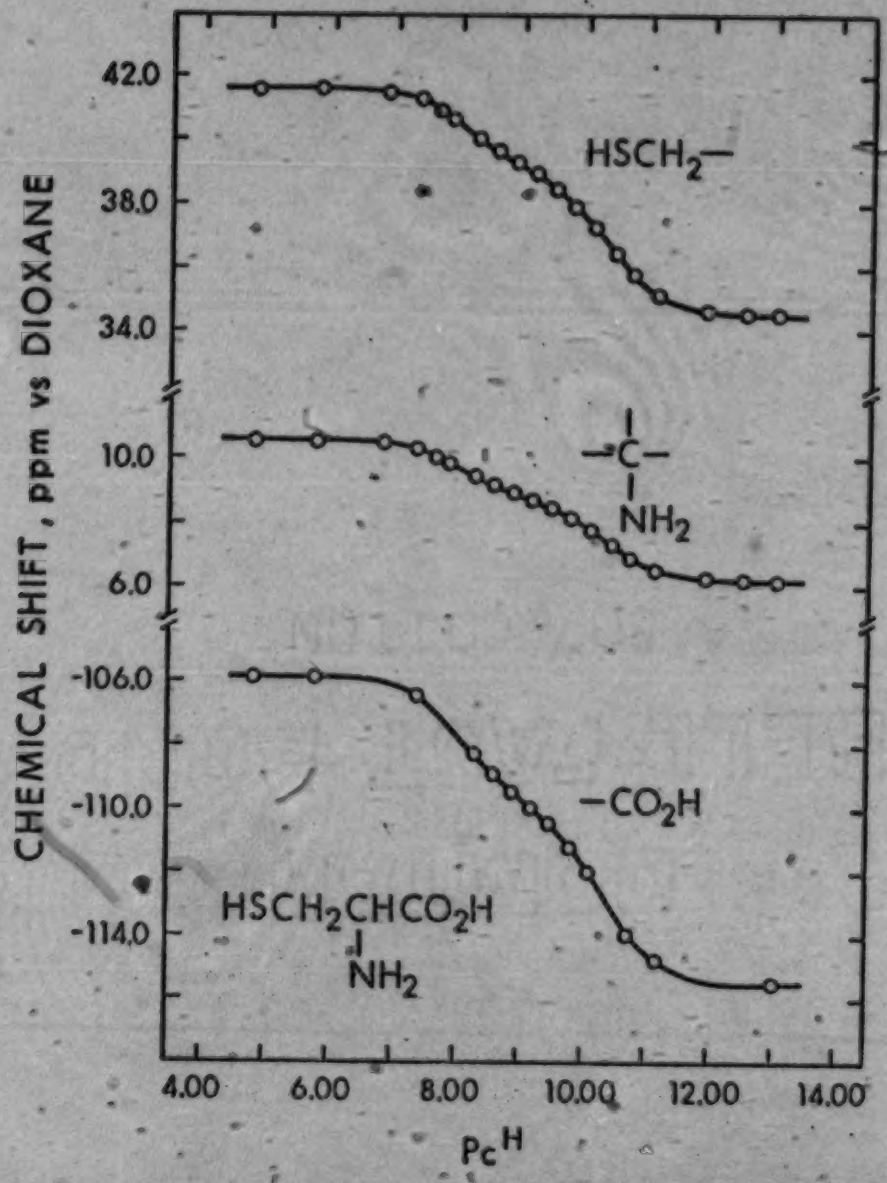


Figure 31: pH dependence of the carbon-13 chemical shifts of the three cysteine carbon atoms in an aqueous solution containing 0.20 M cysteine. 25°C.

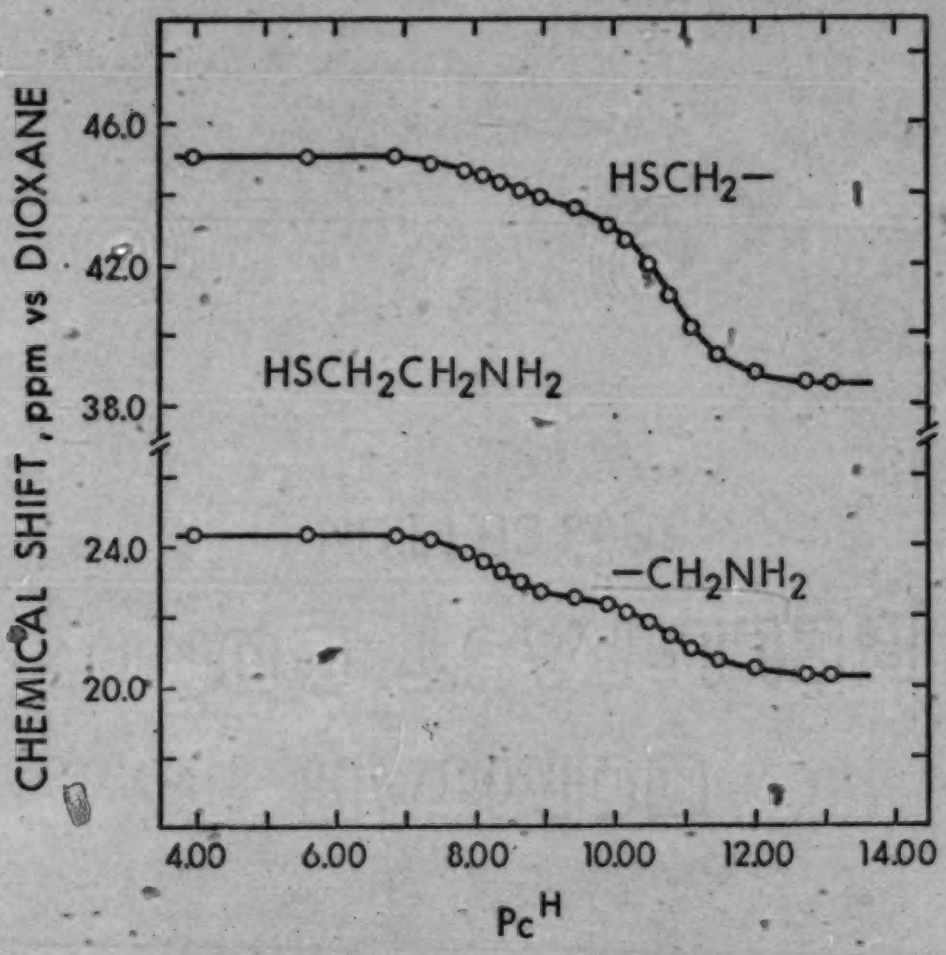


Figure 32: pH dependence of the carbon-13 chemical shifts of the two 2-aminoethanethiol carbon atoms in an aqueous solution containing 0.20 M 2-aminoethanethiol hydrochloride. 25°C.

observing the off resonance decoupled carbon-13 spectrum. In 2-aminoethanethiol, the assignment was made by examining the magnitude of the carbon-13 shifts on deprotonation. Benesch and Benesch (6) have shown by ultraviolet measurements that $K_A^{SH} >> K_A^{NH_3^+}$ and thus that $K_1 = K_A^{SH}$. The assignment was made on the basis of the expected shift when NH_3^+ deprotonates (20). One carbon resonance shifts 2.16 ppm on the second deprotonation while the other shifts 5.24 ppm. The carbon resonance which shifts the most was assigned to the carbon β to the amino group.

The observed shift for a particular carbon atom is the population weighted average of the shifts of that carbon in forms H_2L, HL and L, that is

$$\delta_{OBS} = P_{H_2L}\delta_{H_2L} + P_{HL}\delta_{HL} + P_L\delta_L, \qquad (21)$$

where

$$P_{H_2L} = \frac{[H_2L]}{[H_2L] + [HL] + [L]} \qquad (22)$$

$$P_{HL} = \frac{[HL]}{[H_2L] + [HL] + [L]} \qquad (23)$$

$$P_L = \frac{[L]}{[H_2L] + [HL] + [L]} \qquad (24)$$

The concentrations $[H_2L]$, [HL] and [L] can be expressed in terms of the macroscopic acid dissociation constants:

$$P_{H_2L} = \frac{[H^+]^2}{[H^+]^2 + [H^+]K_2 + K_2K_3} \quad (25)$$

$$P_{HL} = \frac{[H^+]K_2}{[H^+]^2 + [H^+]K_2 + K_2K_3} \quad (26)$$

$$P_{L} = \frac{K_2K_3}{[H^+]^2 + [H^+]K_2 + K_2K_3} \quad (27)$$

and the observed shift can be expressed as

$$\delta_{OBS} = \frac{[H^+]^2\delta_{H_2L} + [H^+]K_2\delta_{HL} + K_2K_3\delta_{L}}{[H^+]^2 + [H^+]K_2 + K_2K_3} \quad (28)$$

Values for δ_{H_2L} and δ_L were obtained from the horizontal portions of the shift versus pH curves at pH 4-6 and pH 13-14 respectively. Values for $[H^+]$ and δ_{OBS} were measured experimentally. Equation (28) was used to evaluate K_2, K_3 and δ_{HL} using a non-linear least squares program. The values of K_2, K_3 and δ_{HL} obtained in this manner were then used to predict the observed shifts. The predicted and observed shifts for cysteine, 2-aminoethanethiol and penicillamine are given in Tables XIV, XV and XVI. Table XVII summarizes the results of these calculations and presents literature values of pK_2 and pK_3 for comparison. In most cases, it is difficult to make comparisons with the literature values for two reasons. First of all, the ionic strength used for our

Table XIV. Calculated and Observed Carbon-13 Shifts for Cysteine

pH[a]	p_cH[b]	ionic strength	C_α shift [c]		C_β shift [c]	
			observed	predicted	observed	predicted
13.00	12.98	0.90	6.20	6.21	34.53	34.56
12.59	12.57	0.90	6.20	6.21	34.58	34.57
11.99	11.97	0.90	6.26	6.26	34.69	34.65
11.20	11.17	0.86	6.53	6.53	35.12	35.09
10.80	10.77	0.83	6.91	6.90	35.82	35.72
10.50	10.46	0.79	7.34	7.31	36.25	36.42
10.21	10.17	0.76	7.71	7.74	37.28	37.20
9.89	9.85	0.73	8.15	8.16	37.98	37.97
9.58	9.53	0.72	8.47	8.47	38.52	38.53
9.31	9.26	0.71	8.69	8.67	38.90	38.89
9.00	8.95	0.70	8.84	8.88	39.22	39.24
8.71	8.66	0.70	9.17	9.11	39.60	39.58
8.39	8.34	0.70	9.44	9.43	40.03	40.04

(Table continued on next page)

Table XIV continued

pH[a]	p_cH[b]	ionic strength	C_α shift[c]		C_β shift[c]	
			observed	predicted	observed	predicted
8.05	8.00	0.70	9.82	9.80	40.57	40.55
7.82	7.77	0.70	9.98	10.03	40.84	40.86
7.50	7.45	0.70	10.25	10.27	41.16	41.19
7.04	6.99	0.70	10.52	10.48	41.54	41.45
6.16	6.11	0.70	10.57	10.58	41.65	41.59
4.70	4.65	0.70	10.62	10.60	41.64	41.61

[a] pH represents the negative logarithm of the activity of the hydrogen ion

[b] p_cH represents the negative logarithm of the concentration of the hydrogen ion

[c] ppm versus dioxane

Table XV. Calculated and Observed Carbon-13 Shifts for 2-aminoethanethiol

pH[a]	pc_H[b]	ionic strength	C_{NH_2} shift[c]		C_{SH} shift[d]	
			observed	predicted	observed	predicted
4.09	3.99	0.2	24.33	24.29	45.16	47.07
5.70	5.60	0.2	24.28	24.29	45.05	45.07
6.97	6.87	0.2	24.28	24.27	45.05	45.02
7.47	7.37	0.2	24.11	24.07	44.88	44.91
7.96	7.86	0.2	23.74	23.75	44.67	44.68
8.21	8.11	0.2	23.52	23.51	44.51	44.51
8.47	8.37	0.2	23.25	23.23	44.34	44.31
8.76	8.66	0.2	22.93	22.94	44.07	44.09
9.05	8.95	0.2	22.66	22.71	43.91	43.90
9.55	9.44	0.21	22.44	22.44	43.59	43.60
10.00	9.90	0.23	22.23	22.21	43.10	43.13
10.27	10.17	0.24	22.01	22.00	42.67	42.67
10.57	10.48	0.27	21.74	21.69	41.97	41.91

(Table continued on next page)

Table XV continued

pH[a]	p_cH[b]	ionic strength	C_{NH_2} shift[c]		C_{SH} shift[d]	
			observed	predicted	observed	predicted
10.87	10.78	0.31	21.36	21.34	41.05	41.04
11.17	11.08	0.34	20.99	21.00	40.19	40.21
11.56	11.47	0.37	20.61	20.69	39.38	39.41
12.09	12.00	0.39	20.45	20.49	38.84	38.88
12.83	12.74	0.40	20.28	20.41	38.57	38.68
13.18	13.09	0.40	20.45	20.40	38.68	38.65

[a] pH represents the negative logarithm of the activity of the hydrogen ion

[b] p_cH represents the negative logarithm of the concentration of the hydrogen ion

[c] shift of the carbon bearing the amino group in ppm versus dioxane

[d] shift of the carbon bearing the sulfhydryl group in ppm versus dioxane

Table XVI. Calculated and Observed Carbon-13 Shifts for Penicillamine.

pH [a]	p_cH [b]	ionic strength	C_α shift [c]		C_β shift [c]		C_{CH_3} shift [c]		C_{CH_3} shift [c]	
			observed	predicted	observed	predicted	observed	predicted	observed	predicted
13.42	13.40	0.90	-2.59	-2.54	19.85	19.87	32.15	32.17	38.30	38.28
12.83	12.81	0.90	-2.54	-2.53	19.91	19.80	32.21	32.18	38.36	38.27
11.92	11.90	0.90	-2.48	-2.41	19.85	19.94	32.15	32.25	38.19	38.23
11.60	11.58	0.88	-2.32	-2.28	19.96	20.02	32.26	32.34	38.14	38.18
11.00	10.97	0.86	-1.72	-1.73	20.34	20.34	32.69	32.69	37.99	37.98
10.70	10.66	0.83	-1.29	-1.31	20.60	20.60	32.96	32.96	37.81	37.83
10.41	10.37	0.79	-0.86	-0.87	20.88	20.86	33.28	33.25	37.71	37.68
10.14	10.10	0.76	-0.54	-0.53	21.09	21.07	33.50	33.47	37.60	37.58
9.83	9.79	0.73	-	-	21.25	21.25	33.66	33.66	37.49	37.51
9.53	9.48	0.72	-	-	21.36	21.37	33.82	33.81	37.49	37.48
9.18	9.13	0.71	-	-	21.47	21.46	33.93	33.96	37.49	37.49
8.93	8.88	0.70	-	-	21.52	21.53	34.09	34.10	37.55	37.55
8.61	8.55	0.70	-	-	21.63	21.65	34.36	34.36	37.65	37.68
8.34	8.29	0.70	-	-	21.79	21.78	34.63	34.65	37.82	37.83

8.02	7.97	0.70	0.86	0.85	21.96	21.96	35.07	35.06	38.09	38.06
7.71	7.66	0.70	1.13	1.12	22.12	22.13	35.44	35.45	38.30	38.29
7.02	6.97	0.70	1.51	1.47	22.39	22.35	36.03	35.94	38.57	38.59
6.54	6.49	0.70	1.56	1.55	-		36.09	36.06	38.63	38.66
5.01	4.96	0.70	1.73	1.60	22.50	22.44	36.25	36.11	38.73	38.70

[a] pH represents the negative logarithm of the activity of the hydrogen ion

[b] p_cH represents the negative logarithm of the concentration of the hydrogen ion

[c] ppm versus dioxane

Table XVII. Calculated Macroscopic Constants

Compound			pK_2 [a]	pK_3 [a]	δ_{HL} [b]	Source
Cysteine	this work:	C_α	8.14	10.35	8.73	-
	this work:	C_β	8.16	10.31	39.06	-
	literature		8.53	10.53	-	(21)
			8.13	10.11	-	(22)
	pH titration [c]		8.26	10.38	-	-
2-aminoethane-thiol	this work:	C_{SH}	8.24	10.73	43.76	-
	this work:	C_{NH_2}	8.25	10.71	22.44	-
	literature		8.19	10.73	-	(23)
penicillamine	this work:	C_α	8.02	10.63	0.03	-
	this work:	C_β	8.00	10.59	21.45	
	this work:	CH_3	8.00	10.68	37.38	
	this work:	CH_3	8.04	10.62	33.84	-

(Table continued on next page)

Table XVII continued

Compound		pK_2 [a]	pK_3 [a]	δ_{HL} [b]	Source
	literature	7.97	10.46	-	(24)
		7.88	10.43	-	(22)
		8.03	10.68	-	(25)

[a] for 2-aminoethanethiol pK_2 and pK_3 represent pK_1 and pK_2

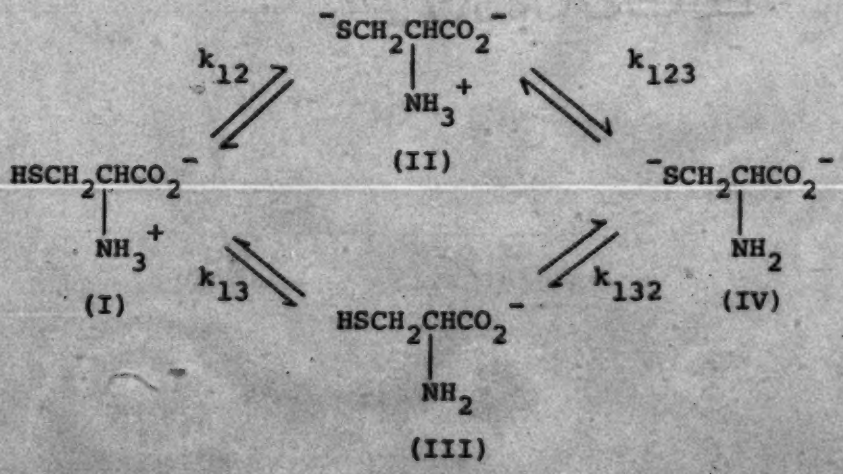
[b] ppm versus dioxane

[c] performed at ionic strength of 0.8

experiments is considerably higher than that used in most of the experiments described in the literature. Secondly, the macroscopic constants reported here are in terms of concentrations whereas most of the literature values are mixed constants containing both activity and concentration terms. For purposes of comparison, a pH titration of cysteine was performed under conditions of ionic strength similar to that of the NMR experiment (μ = 0.8). Values of 8.26 $\pm$ 0.01 and 10.38 $\pm$ 0.02 were obtained for pK_2 and pK_3. These values compare well with those obtained by carbon-13 magnetic resonance.

2. Determination of Microscopic Acid Dissociation Constants from Carbon-13 Chemical Shifts.

At the molecular level, dissociation of the second and third protons from cysteine is described by the following scheme.



The observed shift is a population weighted average of the four forms, that is

$$\delta_{OBS} = P_I\delta_I + P_{II}\delta_{II} + P_{III}\delta_{III} + P_{IV}\delta_{IV} \quad (29)$$

where

$$P_I = \frac{[I]}{[I] + [II] + [III] + [IV]} \quad (30)$$

$$P_{II} = \frac{[II]}{[I] + [II] + [III] + [IV]} \quad (31)$$

$$P_{III} = \frac{[III]}{[I] + [II] + [III] + [IV]} \quad (32)$$

$$P_{IV} = \frac{[IV]}{[I] + [II] + [III] + [IV]} \quad (33)$$

The concentrations of species I - IV can be expressed in terms of the microscopic constants:

$$P_I = \frac{[H^+]^2}{[H^+]^2 + [H^+]k_{12} + [H^+]k_{13} + k_{15}k_{132}} \quad (34)$$

$$P_{II} = \frac{[H^+]k_{12}}{[H^+]^2 + [H^+]k_{12} + [H^+]k_{13} + k_{13}k_{132}} \quad (35)$$

$$P_{III} = \frac{[H^+]k_{13}}{[H^+]^2 + [H^+]k_{12} + [H^+]k_{13} + k_{12}k_{132}} \quad (36)$$

$$P_{IV} = \frac{k_{13}k_{132}}{[H^+]^2 + [H^+]k_{12} + [H^+]k_{13} + k_{13}k_{132}} \quad (37)$$

and the shift can be expressed as

$$\delta_{OBS} = \frac{[H^+]^2\delta_I + [H^+]k_{12}\delta_{II} + [H^+]k_{13}\delta_{III} + k_{13}k_{132}\delta_{IV}}{[H^+]^2 + [H^+]k_{12} + [H^+]k_{13} + k_{13}k_{132}} \quad \ldots (38)$$

which can be rearranged to the form

$$\delta_{OBS} = \frac{\delta_{IV} + \frac{k_{12}\delta_{II} + k_{13}\delta_{III}}{k_{13}k_{132}}[H^+] + \frac{\delta_I}{k_{13}k_{132}}[H^+]^2}{1 + \frac{k_{12} + k_{13}}{k_{13}k_{132}}[H^+] + \frac{1}{k_{13}k_{132}}[H^+]^2} \quad \ldots (39)$$

Equation (31) is of the form

$$\delta_{OBS} = \frac{a + b[H^+] + c[H^+]^2}{1 + d[H^+] + e[H^+]^2} \quad (40)$$

A non-linear least squares analysis of data obtained for a range of hydrogen ion concentrations will yield the coefficients a, b, c, d, and e. However, an infinite number of sets of k_{12}, k_{13}, k_{132}, δ_{II} and δ_{III} will give the same b. In order to resolve the microscopic dissociation constants, values for δ_{II} and δ_{III} are needed.

Equation (38) for the microscopic constants is similar to Equation (28) derived from the macroscopic constants. The shifts δ_{H_2L} and δ_L are equivalent to δ_I and δ_{IV}. The macroscopic product K_2K_3 equals the microscopic product $k_{13}k_{132}$. The coefficient of the $[H^+]$ term in Equation (28) is $K_2\delta_{HL}$, while in Equation (38) it is $k_{12}\delta_{II} + k_{13}\delta_{III}$. Equation (38) can be shown to be exactly equivalent to Equation (28) by the above replacements and the following:

$$\delta_{HL} = P_{II}\delta_{II} + P_{III}\delta_{III}, \tag{41}$$

$$\delta_{HL} = \frac{k_{12}\delta_{II}}{K_2} + \frac{k_{13}\delta_{III}}{K_2}, \tag{42}$$

$$K_2\delta_{HL} = k_{12}\delta_{II} + k_{13}\delta_{III}. \tag{43}$$

An alternative way of obtaining values for the microscopic constants is to use the value δ_{HL} which is obtained for the shift of the monoprotonated species from the evaluation of the macroscopic parameters. Since

$$\delta_{HL} = F_{II}\delta_{II} + F_{III}\delta_{III} \tag{44}$$

where F_{II} and F_{III} represent the fractional concentrations of species II and III and

$$F_{II} + F_{III} = 1, \tag{45}$$

$$F_{II} = \frac{\delta_{HL} - \delta_{III}}{\delta_{II} - \delta_{III}} \qquad (46)$$

Also,

$$\frac{F_{II}}{F_{III}} = \frac{k_{12}}{k_{13}} \qquad (47)$$

Therefore

$$\frac{F_{II}}{F_{III}} = \frac{k_{12}}{K_2 - k_{12}} \qquad (48)$$

and

$$k_{12} = F_{II}K_2 \qquad (49)$$

Thus, if δ_{II} and δ_{III} are known, the microscopic constants can be evaluated directly from δ_{HL}. Attempts were made to determine δ_{II} and δ_{III} using model compounds.

S-methylcysteine was the first model compound used. At pH 5.32, the shifts of the C_α, C_β and C_{CH_3} carbons are 13.16 , 32.04 , and 51.95 ppm respectively, while at pH 12.47 the corresponding shifts are 11.65 , 27.35 and 51.73 ppm. Thus the C_α resonance shifts by 1.51 ppm while the C_β resonance shifted by 4.69 ppm. If one attributes these shifts to the deprotonation of the amino group and assumes that the C_α and C_β resonances of cysteine shift by the same amount when the amino group is de-

protonated, the shifts of species II and III may be predicted. For example, the C_α resonance of cysteine is at 10.59 ppm in species I and 6.20 ppm in species IV, giving a total shift of 4.39 ppm. If 1.51 ppm of this shift is due to the amino deprotonation, the remainder, 2.88 ppm, must be due to the sulfhydryl deprotonation. Thus the C_α shift in species II is 10.59 - 2.88 or 7.71 ppm. In species III, the C_α shift is 10.59 - 1.51 or 9.08 ppm. The shift δ_{HL} for the C_α carbon was found from the non-linear least squares fit to be 8.72 ppm. One obtains a value of 0.263 for F_{II} from Equation (46) and the above shifts.

A similar analysis of the C_β chemical shifts gave a value of 0.920 for F_{II}. This value doesn't agree with the value obtained from the C_α data nor with the known relative acidities of the sulfhydryl and amino groups.

Attempts were made to perform similar calculations using glutathione as the model compound. The cysteinyl residue of glutathione can be considered an N-substituted cysteine and the shifts of the CYS-C_α and CYS-C_β carbon resonances attributed to sulfhydryl deprotonation. The shifts of the intermediate species, II and III, can be calculated using the observed glutathione shifts by the procedure described above for S-methylcysteine. By treating the C_α data in this manner, F_{II} was found to be 0.137, while a value of 0.748 was obtained from the

analysis of the C_β data. Again the agreement is unsatisfactory.

Benesch and Benesch (6) concluded from ultraviolet measurements that the first deprotonation in 2-aminoethanethiol is from the sulfhydryl group and the second from the amino group. They found that there was no overlap or mixing of the deprotonation reactions. Therefore 2-aminoethanethiol can be considered as a model compound and its shifts used to calculate δ_{II} and δ_{III} for cysteine. Since the total shift on deprotonation of both groups is not the same in 2-aminoethanethiol as it is in cysteine, the shifts due to deprotonation of a specific group were estimated by assuming that the fraction of the total shift due to deprotonation of the sulfhydryl group is the same in 2-aminoethanethiol as it is in cysteine. Analyzing the data in this way, one obtains a value of 0.729 for F_{II} from the C_β results. The C_α data showed δ_{II} and δ_{III} both to be less than δ_{HL} so that it was impossible to obtain F_{II} from the C_α shift data.

The results of the above calculations of δ_{II} and δ_{III} for cysteine from the shifts observed in model compounds are summarized in Table XVIII. The values predicted for the ratio R of the sulfhydryl-deprotonated form to the amino-deprotonated form are also included in this table. To estimate the approximate value of R, pH titrations of cysteine and S-methylcysteine were performed

Table XVIII. Predicted Values of δ_{II} and δ_{III} using Model Compounds.

Model Compound	Carbon	δ_{II}^{PRED} [a]	δ_{III}^{PRED} [a]	R[b]
S-methylcysteine	C_α	7.711	9.084	0.36
	C_β	39.248	36.952	11.5
Glutathione	C_α	7.945	8.850	0.16
	C_β	40.045	36.155	2.9
2-aminoethanethiol	C_α	8.545	8.250	-
	C_β	40.205	35.995	2.7

[a] ppm versus dioxane

[b] ratio of sulfhydryl-deprotonated intermediate to amino-deprotonated intermediate.

at an ionic strength of 0.8 which is approximately the ionic strength used in the NMR experiments. Macroscopic constants for pK_2 and pK_3 of cysteine were determined to be 8.26 and 10.38. For S-methylcysteine a value for pK_2 of 8.80 was obtained. By assigning this value to the microscopic constant pk_{13} of cysteine, the microscopic constant pk_{12} was determined to be 8.41. The value of R obtained from these data is 2.48.

C. Discussion

1. Evaluation of Macroscopic Constants From Carbon-13 Chemical Shift Data.

The macroscopic constants obtained for cysteine, penicillamine and 2-aminoethanethiol from carbon-13 chemical shifts indicate that this is a reliable method for determining macroscopic dissociation constants even when the deprotonation reactions overlap. The results listed in Table XVII compare favorably with those available in the literature.

2. Evaluation of Microscopic Dissociation Constants From Carbon-13 Chemical Shift Data.

The evaluation methods described earlier in this chapter indicate that there are difficulties in using carbon-13 chemical shift data as a means of obtaining microscopic dissociation constants when the chemical shift

of the carbon atom is affected by the deprotonation of both groups. In such cases, it is necessary to know the shifts of the two monoprotonated isomers in order to determine the microscopic constants. These shifts cannot be obtained directly since both isomers are always present.

One approach which was tried was the use of model compounds to estimate the shifts of the monoprotonated forms. This approach was not successful, which is not too surprising in view of the assumptions involved in the model compound approach. First of all, carbon-13 shifts are known to be extremely sensitive to conformational changes (26). It is not possible to take into account the differences in the conformations of the model and the actual compound nor the conformational changes that occur on deprotonation.

Another assumption involved in the model compound approach is that the shift of a given carbon resonance upon deprotonation of a certain functional group is always the same no matter what the state of other functional groups. In other words the assumption is that, for a particular carbon atom, $\delta_{II} - \delta_{I}$ equals $\delta_{IV} - \delta_{III}$.

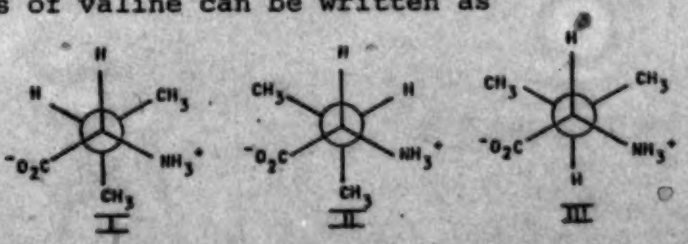
Recently Walters and Leyden (15) used the model compound approach in a determination of the microscopic ionization scheme of cysteine using pmr spectroscopy. Not surprisingly, the results they obtained are in disagreement with the results obtained by other workers using

more direct methods (6,7). Both CMR and PMR methods have the same basic problem: the determination of the shifts of the monoprotonated species, δ_{II} and δ_{III}. Any expression which can be derived to describe the observed shift as a function of the microscopic constants necessarily involves δ_{II} and δ_{III}. Until good estimates of these parameters can be obtained, it is impossible to evaluate the microscopic ionization constants from NMR data.

3. Conformational Dependence of Carbon-13 Shifts.

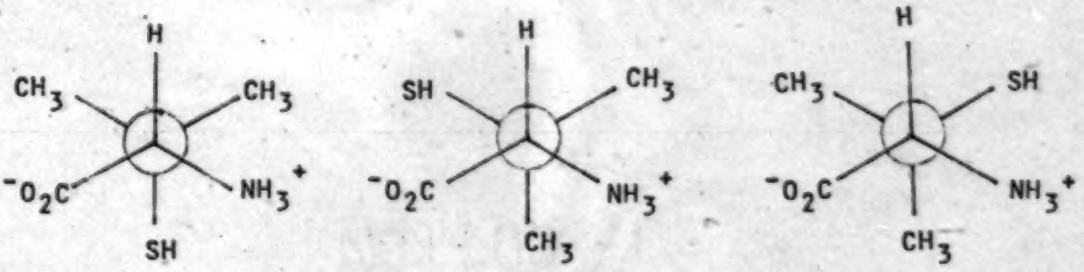
In the results for penicillamine presented in Figure 30, one of the methyl carbons shifts first in one direction as one proton is removed and then in the other direction as the second proton is removed. Presumably this occurs because the major conformational form is different in the deprotonated species from that in the protonated species.

Tran-Dinh et. al. (26) have recently applied carbon-13 magnetic resonance to the problem of determining conformations of the four amino acids: alanine, valine, leucine and isoleucine. Their results for valine are representative and are discussed below. The three conformers of valine can be written as



The position of the carbon-13 resonance for each methyl carbon changes uniquely with pH. One methyl resonance shifts as both the carboxyl and amino groups are deprotonated, while the other methyl resonance remains constant. From this it was concluded that one methyl carbon must be localized in the <COO^-, NH_3^+> zone as in conformer I or II and that the relative proportions of conformers I and II do not vary with pH.

In penicillamine the three conformers are:



I II III

The anomalous shifts shown by the two methyl groups (Figure 30) can be explained in terms of conformational changes as deprotonation proceeds. On going from H_2L to HL^- both methyl groups shift by a similar amount which indicates that both spend some time in the critical <COO^-, NH_3^+> zone. Thus a mixture of conformers II and III exists. On going from HL^- to L^{2-} a change in the conformational population occurs. Only one methyl resonance shifts further downfield on deprotonation suggesting one conformation is predominating, most likely III since in this rotamer the two negatively charged groups, COO^- and S^- are trans to each other.

BIBLIOGRAPHY

1. J.P. Danehy and C.P. Noel, J. Amer. Chem. Soc., 82, 2511 (1960).
2. J. Bjerrum, Chem. Rev., 46, 381 (1950).
3. J.T. Edsall and J. Wyman, "Biophysical Chemistry", Academic Press, New York, N.Y., 1958, p. 487.
4. R.L. Ryklan and C.L.A. Schmidt, Arch. Biochem., 5, 89 (1944).
5. M.A. Grafius and J.B. Neilands, J. Amer. Chem. Soc., 77, 3389 (1955).
6. R.E. Benesch and R. Benesch, J. Amer. Chem. Soc., 77, 5877 (1955).
7. E.L. Elson and J.T. Edsall, Biochemistry, 1, 1 (1962).
8. D.P. Wrathall, R.M. Izatt and J.J. Christensen, J. Amer. Chem. Soc., 86, 4779 (1964).
9. E. Coates, C.G. Marsden and B. Rigg, Trans. Faraday Soc., 65, 3032 (1969).
10. E.W. Wilson and R.B. Martin, Arch. Biochem. Biophys., 142, 445 (1971).
11. E. Grunwald, A. Loewenstein and S. Meiboom, J. Chem. Phys., 27, 641 (1957).
12. A. Loewenstein and J.D. Roberts, J. Amer. Chem. Soc., 82, 2705 (1960).
13. R.B. Martin, J. Phys. Chem., 65, 2053 (1961).
14. N.E. Rigler, S.P. Bag, D.E. Leyden, J.L. Sudmeier and C.N. Reilley, Anal. Chem., 37, 872 (1965).

15. D.B. Walters and D.E. Leyden, Anal. Chim. Acta, 72, 275 (1974).

16. D.L. Rabenstein, J. Amer. Chem. Soc., 95, 2797 (1973).

17. H.S. Creyf, L.C. van Poucke and Z. Eeckhart, Talanta, 20, 973 (1973).

18. L. Meites, "Handbook of Analytical Chemistry", McGraw-Hill, New York, N.Y., 1963, pp. 1-8.

19. D.W. Marquardt, J. Soc. Indust. Appl. Math., 11, 431 (1963).

20. M. Christl and J.D. Roberts, J. Amer. Chem. Soc., 94, 4565 (1972).

21. D.J. Perkins, Biochem. J., 55, 649 (1953).

22. G.R. Lenz and A.E. Martell, Biochemistry, 3, 745 (1964).

23. Y. Tsuchitani, T. Ando and K. Ueno, Bull. Chem. Soc. Japan, 36, 1534 (1963).

24. E.J. Kuchinkos and Y. Rosen, Arch. Biochem. Biophys., 97, 370 (1962).

25. D.D. Perrin and I.G. Sayer, J. Chem. Soc. (A), 53 (1968).

26. S. Tran-Dinh, S. Fermandjian, E. Sala, R. Mermet-Bouvier, M. Cohen and P. Fromageot, J. Amer. Chem. Soc., 96, 1484 (1974).